Interaction of Nutrient Availability and Endurance Training on Skeletal Muscle Adaptation in Trained Humans

A thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

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School of Medical Sciences
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RMIT University
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RESEARCH OUTCOME

The following publications have resulted from the work undertaken in this thesis:

Peer-reviewed articles:


Manuscript in preparation:


Published abstracts:


DECLARATION

I, the candidate, Wee Kian Yeo, certify that:

a) the AMPK signaling data in Chapter 3 was obtained in conjunction with Dr. Sarah Lessard and Dr. Zhiping Chen at St. Vincent’s Institute, Melbourne.

b) the mitochondrial enzymatic activities and the mtDNA data in Chapter 4 were obtained in conjunction with Dr. Andrew Carey at the Exercise Metabolism Laboratory, RMIT University.

c) the HDAC5, p-ATF2 and p-CREB data in Chapter 5 were obtained in conjunction with Dr. Sean McGee at the University of Melbourne.

d) except where due acknowledgement has been made, the work is that of the candidate alone;

e) the work has not been submitted previously, in whole or in part, to qualify for any other academic award;

f) the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program;

g) any editorial work, paid or unpaid, carried out by a third party is acknowledged;

h) ethics procedures and guidelines have been followed.

Wee Kian Yeo

March 2009
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ABBREVIATIONS

20SS  20-min steady state ride at 70% of VO_{2peak}
60SS  60-min steady state ride at 70% of VO_{2peak}
ACC   acetyl-CoA carboxylase
ADP   adenosine diphosphate
AMP   adenosine monophosphate
AMPK  5’ adenosine monophosphate kinase
ANOVA analysis of variance
AT    aerobic training (100-min steady state cycle at 70% VO_2 peak)
ATF2  activating transcription factor 2
ATP   adenosine triphosphate
β-HAD β-hydroxyacyl-CoA dehydrogenase
BM    body mass
CaMK  calcium calmodulin dependant protein kinase
CAMKK calcium calmodulin dependant protein kinase kinase
CHO   carbohydrate
COX II cytochrome C oxidase subunit II
COX IV cytochrome C oxidase subunit IV
CPT1  carnitine palmitoyltransferase 1
CRE   cyclic AMP response element
CREB  cyclic AMP response element binding protein
CS    citrate synthase
DNA   deoxyribose nucleic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>FA</td>
<td>fatty acids</td>
</tr>
<tr>
<td>FABP&lt;sub&gt;pm&lt;/sub&gt;</td>
<td>plasma membrane fatty acid binding protein</td>
</tr>
<tr>
<td>FAT-adapt</td>
<td>fat adaptation and carbohydrate restoration</td>
</tr>
<tr>
<td>FAT/CD36</td>
<td>fatty acid translocase</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acids</td>
</tr>
<tr>
<td>GLUT4</td>
<td>glucose transporter 4</td>
</tr>
<tr>
<td>HDAC5</td>
<td>histone deacetylase 5</td>
</tr>
<tr>
<td>HIGH</td>
<td>group that performed HIT in the face of normal muscle glycogen</td>
</tr>
<tr>
<td>HIT</td>
<td>high intensity interval training (8 x 5 min work bouts at maximal effort)</td>
</tr>
<tr>
<td>LOW</td>
<td>group that performed HIT in the face of low muscle glycogen</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEF2</td>
<td>myocyte enhancer factor 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>mtDNA</td>
<td>mitochondrial DNA</td>
</tr>
<tr>
<td>NRF</td>
<td>nuclear respiratory factor</td>
</tr>
<tr>
<td>PDH</td>
<td>pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>peroxisome proliferator activated receptor &lt;i&gt;gamma&lt;/i&gt; co-activator-1 &lt;i&gt;alpha&lt;/i&gt;</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PPO</td>
<td>peak power output</td>
</tr>
<tr>
<td>RER</td>
<td>respiratory exchange ratio</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline with tween</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>TG</td>
<td>triglyceride</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2max&lt;/sub&gt;</td>
<td>maximal oxygen uptake</td>
</tr>
<tr>
<td>VO&lt;sub&gt;2peak&lt;/sub&gt;</td>
<td>peak oxygen uptake</td>
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</table>
Abstract

Nutrient availability and exercise training are two potent modulators of skeletal muscle adaptation. While the effects of nutrient provision or training alone on muscle adaptation have been well researched, the interactive effects of these two stressors on whole-body metabolism and skeletal muscle adaptation are less well understood, especially in well-trained individuals. Furthermore, until recently, little work has been undertaken to determine some of the mechanisms that underlie the changes in muscle metabolism that result from the interaction of nutrient and contractile status. Accordingly, the primary aims of the experiments undertaken for this thesis were: 1) to enhance our understanding of how skeletal muscle nutrient availability interacts with exercise training to promote changes in muscle and whole-body metabolism in well-trained athletes, and 2) to determine potential mechanisms that might underlie some of these adaptations.

The first experiment (described in Chapter Three) was undertaken to determine the effects of a dietary periodisation strategy termed “fat adaptation and CHO restoration”, on subsequent changes in skeletal muscle fuel storage profile, whole-body metabolism and the potential mechanisms underlying these changes in well-trained humans. Utilising a randomised, crossover design, eight well-trained cyclists/triathletes underwent two 7-day trials separated by a 2-wk washout period. The interventions consisted of prescribed supervised endurance training while consuming either a high-fat diet for 5-days (4.6 g/kg/day fat, 68% of energy) followed by 1-day of high CHO restoration (FAT-adapt) or an isoenergetic high CHO diet for 6-days (HCHO; 10.3 g/kg/day and 70% of energy). Muscle samples (~100-150 mg) were taken from the vastus lateralis at rest and after 60-min steady state cycling at 70% VO$_{2peak}$ (60SS) undertaken on day-7.
of each intervention. Resting muscle glycogen content was not different between treatments (HCHO, 694 ± 55 vs. FAT-adapt, 547 ± 40 μmol/g dry wt.), but muscle triglyceride (TG) levels were higher after FAT-adapt (69 ± 10.2 vs. 39 ± 3.4 μmol/g dry wt.; P < 0.01). Resting 5’ AMP-activated protein kinase (AMPK) -α1 and -α2 activity were higher after FAT-adapt (P < 0.05), while the phosphorylation of AMPK’s downstream target, acetyl-CoA carboxylase (pACC at Ser221), tended to be elevated after FAT-adapt (P = 0.09). Both the respiratory exchange ratio (RER; 0.87 ± 0.004 vs. 0.93 ± 0.004; P < 0.01) and muscle glycogen utilisation (179 ± 35 vs. 264 ± 45 μmol/g dry wt., P < 0.05) were lower during exercise after FAT-adapt. Exercise (60SS) increased AMPK-α1 activity after HCHO (P < 0.05) but not FAT-adapt. Exercise was associated with an increase in pACC at Ser221 for both dietary treatments (P < 0.05), with post exercise pACC Ser221 higher after FAT-adapt (P < 0.05). Resting muscle glycogen concentration was associated with AMPK-α1 activity (r = -0.51; P < 0.05) while resting muscle TG concentration was related to AMPK-α2 activity (r = 0.53; P < 0.05). In conclusion, compared with HCHO, FAT-adapt increased resting muscle TG stores and resting AMPK-α1 and -α2 activity. FAT-adapt also resulted in higher rates of whole-body fat oxidation, reduced muscle glycogenolysis, and attenuated the exercise-induced rise in AMPK-α1 and AMPK-α2 activity compared with HCHO. These results demonstrate that AMPK-α1 and AMPK-α2 activity and fuel selection in skeletal muscle in response to exercise can be manipulated by diet and/or the interactive effects of diet and exercise training.

The second experiment described in Chapter Four was undertaken to determine the effects of a cycle training programme in which selected training sessions (50%) were commenced with low muscle glycogen content on training capacity and subsequent endurance performance, rates of
whole-body substrate oxidation during submaximal exercise, and several mitochondrial enzymes and signalling proteins associated with training adaptation. Seven endurance-trained cyclists/triathletes trained daily (HIGH) alternating between 100-min steady-state aerobic rides (AT) one day, followed by a high-intensity interval training session (HIT; 8 x 5 min at maximum self-selected effort) the next day. Another seven subjects matched for age, peak oxygen consumption (VO₂peak) and training history trained twice every second day (LOW), first undertaking AT, then 1–2 hr later, the HIT. Training power output (W) was monitored during each session and the respective training schedules were maintained for 3-wk. Forty-eight hours before and after the first and last training session, all subjects completed a 60SS followed by a 60-min performance trial. Muscle biopsies were taken before and after 60SS, with rates of substrate oxidation determined at regular intervals throughout this ride. Performance was determined as the highest average power output (W) maintained during the ride. Resting muscle glycogen concentration (412 ± 51 vs. 577 ± 34 μmol/g dry wt.), rates of whole body fat oxidation during 60SS (1,261 ± 247 vs. 1,698 ± 174 μmol/kg/60 min), the maximal activities of citrate synthase (45 ± 2 vs. 54 ± 1 mmol/kg dry wt./min), and β-hydroxyacyl-CoA dehydrogenase (18 ± 2 vs. 23 ± 2 mmol/kg dry wt./min) along with the total protein content of cytochrome c oxidase subunit IV were increased only in LOW after the 3-wk intervention (all P < 0.05). Mitochondrial DNA content and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) protein levels were unchanged for both groups after training. Self-selected training intensity was lower in week 1 (P < 0.05) and tended to be lower in week 2 (P = 0.06) in LOW, but was not different for both groups in week 3. Cycling performance improved by ~ 10% in both LOW and HIGH. It is concluded that compared with training daily, training twice every second day compromised high-intensity training capacity. While selected muscle markers of training adaptation were enhanced
with twice-a-day training, the performance of a 1-hr time trial undertaken after a 60-min steady-state ride was similar after once daily or twice every second day training programmes.

The final experiment in this thesis (Chapter Five) was undertaken in an attempt to elucidate potential mechanisms underlying the enhanced training adaptations observed after performing selected training sessions with low muscle glycogen concentration. Six endurance-trained cyclists/triathletes performed an AT on day-1 and HIT 24 h later (HIGH). Another six subjects matched for age, VO$_{2peak}$ and training history performed AT on day 1 then 1-2 h later, HIT (LOW). Muscle biopsies were taken before and after HIT. AMPK phosphorylation increased in both groups after HIT ($P < 0.05$) but to a greater extent in LOW compared with HIGH ($P < 0.05$). Despite the augmented AMPK response in LOW after HIT, the localisation and phosphorylation state of selected AMPK downstream substrates were not different between groups. Phosphorylation of p38 MAPK was unchanged for both groups before and after HIT sessions. Phosphorylation of CAMKII-β$_m$ isoform was similar before HIT in both groups but increased significantly only in HIGH after HIT. There were no effects of treatment or time on the phosphorylation of CAMKII-δ/γ isoform or when all three of the CAMKII isoforms were quantified collectively. Taken collectively, these results suggested that AMPK was phosphorylated to a greater extent when performing HIT sessions with low muscle glycogen concentration and the enhanced adaptations seen in the previous study (Chapter Four) were likely to be mediated by AMPK and/or other unidentified downstream targets.

In summary, the results of the studies undertaken for this thesis provide novel insights into the mechanisms by which the manipulation of nutrient availability and subsequent muscle fuel
storage profile interact with endurance exercise training to promote changes in skeletal muscle metabolism and adaptation in well-trained athletes. A dietary periodisation strategy consisting of fat adaptation and CHO restoration was shown to increase resting muscle TG and glycogen content, with these changes in fuel stores being associated with an isoform specific activation of AMPK. The increase in AMPK activity after fat adaptation, coupled with the increased phosphorylation status of AMPKs downstream target, ACC, provides evidence of a mechanistic link between muscle nutrient availability and the subsequent metabolic responses. However, while it is tempting to attribute a causal role for altered muscle substrate availability (i.e. glycogen and/or triglyceride stores) on subsequent changes in AMPK signalling, it should be acknowledged that differences in AMPK responses could also be due to the well-trained subjects performing prolonged, strenuous training on a high-fat diet (i.e., the interactive effects of diet and exercise). To further investigate the links between muscle fuel stores and training adaptation, well-trained subjects undertook a chronic (3-wk) intervention in which they commenced ~50% their training sessions with either normal or reduced muscle glycogen content. Selected markers of training adaptation were enhanced with twice-a-day versus daily training. Again, AMPK was elevated to a greater extent when subjects trained with low compared to normal glycogen content, although selected downstream targets were unaffected by either diet-training regimen. It would seem safe to conclude that the changes in AMPK activation observed in these studies could be due to altered fuel status (i.e., increased muscle lipid availability, reduced muscle glycogen content), a greater adaptive response when training with low muscle glycogen availability, or most likely, the interactive effects of both these factors.
Chapter One

Literature Review
1.1 Nutritional intervention: performance versus training adaptation.

Nutrient availability has long been recognised as one of the most potent modulators of skeletal muscle adaptation. The manipulation of dietary macronutrient intake lasting as short as 2-5 days has been shown to have marked effects on substrate stores (Zderic, et al., 2004) and skeletal muscle gene expression (Arkinstall, et al., 2004; Cameron-Smith, et al., 2003; Jump and Clarke, 1999). This in turn affects not only resting energy metabolism, but also metabolic flux and fuel utilisation during subsequent exercise (Coyle, et al., 2001; Horowitz, et al., 1997; 1999). While the effects of training and diet alone on skeletal muscle adaptation and exercise performance have been studied extensively, the interactive effects of diet and training on skeletal muscle adaptation have received less attention and the regulation of the cellular and molecular processes of adaptation to these two stimuli are largely unknown (Hawley, et al., 2006).

It is commonly accepted that adaptation to training requires optimal muscle nutrient reserves prior to and during recovery from training (Burke, et al., 2004; Hawley, et al., 2006; Jentjens, et al., 2003). In this regard, a high carbohydrate (CHO) diet (~80% of energy; >6g CHO/kg body mass) has been associated with elevated muscle and liver glycogen concentration and enhancement of subsequent exercise performance compared to a CHO restricted diet (Bergstrom, et al., 1967). Indeed, the current consensus is that a high CHO diet enables an athlete to train longer, harder and faster and thus achieves a superior training response compared to a diet low in CHO (Coyle, 2000; Hansen, et al., 2005; Hawley, et al., 2006). However this paradigm does not consider the long unsolved question of whether it is a lack or a surplus of a substrate (i.e. CHO) that triggers skeletal muscle adaptation in response to training.
While the role of high CHO diet on metabolism and exercise capacity has been well researched, it is currently unknown if this dietary strategy provides a superior training stimulus for skeletal muscle adaptation compared to a diet low in CHO. Indeed, it has recently been shown that commencing exercise with low muscle glycogen concentration increases the transcription rate of several genes and proteins involved in the training response/adaptation (Febbraio, et al., 2002; Furuyama, et al., 2003; Keller, et al., 2001; Pilegaard, et al., 2002; Steinberg, et al., 2006; Wojtaszewski, et al., 2003). In rats and cell lines, raising plasma free fatty acid (FFA) levels activates several signalling proteins involved in fatty acid oxidation (Garcia-Roves, et al., 2007) and mitochondrial biogenesis (Garcia-Roves, et al., 2007; Watt, et al., 2006), as well as increases the expression of genes encoding fatty acid oxidation enzymes and mitochondrial citrate cycle and respiratory chain enzymes (Garcia-Roves, et al., 2007). Furthermore, the increase in transcriptional regulation of several metabolic genes after exercise was sustained/enhanced through 8-24 hr when subjects were given a low CHO diet while a high CHO diet reversed the activation of these genes in 5-8 hr after exercise (Pilegaard, et al., 2005).

Recently, Baar & McGee (2008) postulated that the classic progressive overload principle for exercise training adaptation incorporating exercise frequency, intensity and duration is no longer adequate. They suggested that the “principle of optimal nutrition” needs to be incorporated into modern training programmes, and that training should be optimised by periodising nutrient intake according to the demands and needs of different phases of preparation. In support of this theory, Stellingwerff et al. (2007a) provide specific dietary intake recommendations to optimise training adaptation for different phases of training for middle-distance runners (Table 1.1). In accordance
to the demands and goals of different training phases, Stellingwerff et al., (2007a) recommended that the total energy intake and percentage of fat from total daily energy intake (%FAT) be reduced progressively from the general preparation phase through to the competition phase (Table 1.1). In contrast, the percentage of CHO from total daily energy intake (%CHO) should increase progressively from the general preparation phase through to the competition phase (Table 1.1). It was recommended that during the transition phase, both %CHO and total daily energy intake be tapered while %FAT was recommended to be increased (Table 1.1).

However, ‘dietary periodisation’ is certainly not a new concept: the classical carbohydrate loading strategy of Bergstrom, et al. (1967) was probably the first attempt to manipulate exercise/nutrient intake to optimise muscle fuel stores and exercise performance. In addition, more than a decade ago, Hawley and Hopkins (1995) proposed that dietary periodisation should be utilised to optimise training stimuli and the authors (Hawley and Hopkins, 1995) suggested that both CHO and fat availability to be modified to enhance the aerobic glycolytic and lypolytic power systems respectively.

It is apparent that while the role of a high CHO diet in increasing muscle glycogen stores and enhancing endurance performance is firmly established, less is known regarding manipulating chronic CHO availability during training. In addition, apart form manipulating CHO availability, there seems to be a possibility for fat to be used in conjunction with exercise training to modify training response (Hawley and Hopkins, 1995). Nevertheless, evidence to support this claim is still lacking and the mechanistic explanation for this nutrient-training interaction remains unknown.
Table 1.1  Daily macronutrient intake recommendations for a 70-kg athlete during different yearly training phases.

<table>
<thead>
<tr>
<th>Training phase</th>
<th>General prep.</th>
<th>Specific prep.</th>
<th>Competition</th>
<th>Transition/R&amp;R</th>
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<tr>
<td></td>
<td>Light</td>
<td>Heavy</td>
<td>Light</td>
<td>Heavy</td>
</tr>
<tr>
<td>km · week(^{-1})</td>
<td>&lt;100 km</td>
<td>&gt;150 km</td>
<td>&lt;80 km</td>
<td>&gt;130 km</td>
</tr>
<tr>
<td>h · week(^{-1})</td>
<td>5–8 h</td>
<td>10 h+</td>
<td>4–7 h</td>
<td>6–9 h</td>
</tr>
<tr>
<td>Training intensity</td>
<td>Low</td>
<td>Moderate to high</td>
<td>Tapered training volume and intense racing</td>
<td>Very low to complete rest</td>
</tr>
</tbody>
</table>

| Recommended daily macronutrient intake (g · kg\(^{-1}\) · day\(^{-1}\)) |
|-----------------|----------------|----------------|----------------|----------------|
| CHO             | 7              | 10             | 7              | 10             | 7              | 10             | 4              | 6              |
| FAT             | 1.5            | 2              | 1              | 1.5            | 0.8            | 1.2            | 1              | 1.5            |
| PRO             | 1.5            | 1.7            | 1.5            | 1.7            | 1.2            | 1.5            | 0.8            | 1.2            |

| Percent of total daily energy intake |
|-----------------|----------------|----------------|----------------|----------------|
| %CHO            | ~60%           | ~66%           | ~70%           | ~57%           |
| %FAT            | ~28%           | ~22%           | ~18%           | ~32%           |
| %PRO            | ~12%           | ~12%           | ~12%           | ~11%           |

| Total daily energy intake |
|-----------------|----------------|----------------|----------------|----------------|
| kJ              | ~13900         | ~18900         | ~12600         | ~17600         | ~11700         | ~16600         | ~8200          | ~12400         |
| kcal            | ~3300          | ~4500          | ~3000          | ~4200          | ~2800          | ~4000          | ~2000          | ~2900          |

Prep, preparation; R&R, rest and recovery; CHO, carbohydrate; PRO, protein; [reproduced from Stellingwerff et al. (2007a)].
1.2 Overview: Endurance training and skeletal muscle adaptation

Exercise training is the most potent modulator of skeletal muscle adaptation. Performing a single bout of prolonged endurance exercise provides significant challenges to skeletal muscle metabolic homeostasis. In sedentary individuals, an acute bout of submaximal exercise results in marked increases in the concentrations of adenosine monophosphate (AMP), inorganic phosphate and lactate while concomitantly decreasing the concentrations of adenosine triphosphate (ATP), creatine phosphate (PCr) and muscle substrate stores (i.e. glycogen and triglyceride). However, performing repeated bouts of endurance activity (i.e. exercise training) reduces these perturbations in metabolic homeostasis and subsequently enables individuals to perform exercise of a similar intensity for a longer period of time.

It was initially thought that the increased capacity to perform submaximal prolonged endurance exercise was exclusively due to the cardiovascular adaptations to endurance training which allow more oxygen (O\textsubscript{2}) to be delivered to working muscles (Holloszy and Coyle, 1984). Central to this premise was that the increased O\textsubscript{2} supply resulting from the enhanced cardiorespiratory function would reduce the intramyocellular ‘hypoxic’ environment during subsequent muscular contractions to allow an individual to exercise at similar intensity for longer period of time (Holloszy and Coyle, 1984). However, if this was the case, one would expect the O\textsubscript{2} consumption during submaximal prolonged exercise at a similar absolute intensity to be higher after training. In contrast, it was reported that O\textsubscript{2} consumption during submaximal exercise was the same in the trained and untrained states (Hagberg, et al., 1980; Hickson, et al., 1978). This observation has prompted exercise scientists to believe that the main mechanisms underlying the improved capacity to performed prolonged submaximal exercise observed after endurance training to reside
in skeletal muscle, and in particular the mitochondria. Indeed, in his seminal work using rats, Holloszy (1967) showed that endurance training is a powerful stimulator for mitochondrial adaptations in skeletal muscle. In that study (Holloszy, 1967), rats were subjected to three months of progressive endurance training where running speed and duration were gradually increased. Remarkably, Holloszy (1967) reported that submaximal running time to exhaustion was improved by over 600% (from ~30 min to ~190 min) after this training regimen. This enhanced endurance ability was also accompanied by substantial increases in mitochondrial enzymatic activities and protein concentrations and subsequently higher capacity of respiratory control and oxidative phosphorylation (Holloszy, 1967).

These enhanced mitochondrial adaptations in response to endurance training (termed mitochondrial biogenesis), were later confirmed in humans by many investigators. Both cross-sectional and longitudinal studies in humans revealed that well trained endurance athletes demonstrated higher mitochondrial volume density (Hoppeler, et al., 1973; Morgan, et al., 1971), and increased levels of mitochondrial enzymes involved not only in fatty acid transport (Costill, et al., 1979; Jong-Yeon, et al., 2002) and oxidation (Chi, et al., 1983; Costill, et al., 1979; Jansson and Kaijser, 1977; Schantz, et al., 1983), but also enzymes involved in the citrate cycle (Chi, et al., 1983; Gollnick, et al., 1972; Schantz, et al., 1983) and respiratory chain (Costill, et al., 1979; Gollnick, et al., 1973; Jansson and Kaijser, 1977; Morgan, et al., 1971). These enhanced mitochondrial and enzymatic adaptations subsequently resulted in an enhanced capacity to oxidise fatty acids (FA), thus permitting a greater reliance on fat to fuel oxidative metabolism while concomitantly attenuating endogenous CHO utilisation (Holloszy and Coyle, 1984). Indeed, one of the ‘classic’ adaptations to endurance training is a lower respiratory exchange ratio (RER),
indicating a shift from CHO- to fat-based fuels in both males (Brooks and Mercier, 1994; Hurley, et al., 1986; Martin, et al., 1993) and females (Stisen, et al., 2006). In addition, the mitochondrial enzyme β-hydroxyacyl-CoA dehydrogenase (β-HAD) has also been shown to be significantly correlated with the rate of whole-body fatty acid oxidation during moderate exercise (Stisen, et al., 2006). Furthermore, in human skeletal muscle, exercise training has been shown to increase rates of fat oxidation by ~24% and this was accompanied by a similar increased in FAT/CD36 mRNA and protein content. Compared to untrained humans, endurance trained humans have been shown to utilise more muscle triglyceride (Helge, et al., 2006; Hurley, et al., 1986) while concomitantly reducing muscle glycogen utilisation (41% lower) during exercise (Hurley, et al., 1986).

Taken collectively, these results indicated that endurance training is a powerful stimulator for mitochondrial biogenesis in human skeletal muscle. These increases in mitochondrial morphological and enzymatic adaptations confer an individual greater ability to utilised FA to fuel prolonged exercise. As a result, the depletion of muscle glycogen is delayed and the individual is able to perform similar endurance activity at similar absolute intensity for longer period of time after endurance training.
1.3 **Nutrient-training strategies to enhance training response**

While it is well known that endurance training promotes mitochondrial adaptations that result in marked increases in rates of whole-body fat oxidation, several dietary protocols are capable of promoting further changes in muscle function that augment the oxidation of lipid-based fuels (i.e. muscle TG and blood-borne lipids). These strategies are termed ‘nutrient-training’ strategies in this thesis because they involve the coupling of both nutrient availability and endurance training to promote the intended training response. In this regard, nutrient availability is often modified by either altering the dietary intake profile, or by changing the training schedule which subsequently result in changes in substrate storage profile with the aim of modifying the training stimulus. Chief among these strategies are fat adaptation with and without CHO restoration, training in the face of low muscle glycogen and commencing training in the ‘fasted’ state.

1.3.1 **Fat adaptation**

*Background*

While glycogen storage in human skeletal muscle and liver is limited, lipid storage in muscle and particularly adipose tissue is abundant, with absolute amounts being sufficient for many hours/days of continuous exercise even in the leanest of athletes (Burke and Hawley, 2002). Logically, one would assume that a training method that increases the rate of fat oxidation during exercise to a level beyond that which has already been achieved by training would enhance endurance capacity. Both acute and chronic modification of the fat and CHO content of the diet have been long known to result in altered proportions of substrate oxidation both at rest and during exercise (Burke and Hawley, 2002; Helge, 2000; Helge, 2002; Krogh and Lindhard, 1920).
One such dietary-training intervention termed “fat adaptation” is when an athlete consumes a high-fat, low-CHO diet while undertaking an endurance training programme in order to promote higher rates of fat utilisation during exercise (protocols to be discussed in subsequent section).

Fat feeding strategies have a long history and since the beginning of last century, studies have been performed to investigate the role of dietary fat manipulation with an aim to link endurance performance and substrate oxidation (Helge, 2000). Helge (2000) reviewed a number of studies that investigate the effects of short- and long-term fat adaptation on metabolism and performance and concluded that while fat adaptation is able to improve substrate utilisation during exercise, performance is often attenuated, or in the best case scenario, maintained (Helge, 2000). However, since then, there have been several studies that have found a performance benefit of fat adaptation strategies in well-trained subjects, although these results are perhaps best explained by individuals who are ‘responders’ (i.e. who can benefit from fat adaptation strategies), while others are ‘non-responders’ (Table 1.2). Indeed, in a recent meta analysis to examine the effects of high fat or high CHO diets on endurance performance, Erlenbusch et al., (2005) showed that there are great differences in the effect sizes between trained and untrained humans in studies investigating high fat diets and performance, indicating that trained individuals possess the metabolic machinery to tolerate the effects of high fat diet better than the untrained. These authors (Erlenbusch, et al., 2005) suggested that trained subjects should be studied to increase the likelihood of a high fat effect on performance. Clearly, there appears to be a need to re-visit this topic of interest using well-trained subjects, and the potential mechanisms underlying the alterations in muscle metabolism that result from fat adaptation strategies. In the context of this thesis, such mechanistic links may provide an indication of the effects of fat-adaptation strategies.
in modulating skeletal muscle adaptation in well-trained humans. The following section summarises the potential mechanisms that may underlie the adaptations seen with fat adaptation strategies in well-trained athletes.

**Fat adaptation: potential mechanisms of action**

Fat adaptation strategies dramatically increase whole body rates of fat oxidation and attenuate muscle glycogen utilisation during submaximal exercise in already well-trained athletes (Table 1.2). These alterations in substrate metabolism during exercise are believed to be due to changes residing in skeletal muscle and involve processes associated with either, fat and CHO oxidation at the level of substrate transport at the sarcolemma, substrate storage, breakdown of FA’s or glucose to acetyl-CoA, or mitochondrial transport. In this regard, a primary means for transport of fatty acids into contracting skeletal muscle cells is via the fatty acid transporters FAT/CD36 and plasma membrane fatty acid binding protein (FABP<sub>pm</sub>). Five days of fat adaptation was shown to increase FAT/CD36 mRNA and protein content in well-trained subjects (Cameron-Smith, et al., 2003; Table 1.2). Another fatty acid transporter across sarcolemma investigated in that study (Cameron-Smith, et al., 2003), FABP<sub>pm</sub> was however unchanged, suggesting that FAT/CD36 is more sensitive to changes in dietary fat content than the FABP<sub>pm</sub> and that the improved FA transport across sarcolemma through FAT/CD36 might be one of the mechanisms by which fat adaptation strategies increased fat oxidation during exercise.

In the muscle cells, long chain FA’s first require transport into mitochondria before they can be further broken down prior to oxidation. This process requires transport via mitochondrial carnitine palmitoyltransferase 1 (CPT1). CPT1 activity was found to be increased after both 15
(Goedecke, et al., 1999) and 28 days (Fisher, et al., 1983) of fat adaptation, although a later study (Cameron-Smith, et al., 2003) found that the mRNA and protein abundance of CPT1 was unchanged after a shorter (5 days) intervention on a high fat diet (Table 1.2).

In order to increase the rates of whole-body fat oxidation during exercise, the increase in FA transport across sarcolemma and the mitochondrial membrane should theoretically be coupled with an enhanced ability to oxidised fat. In this regard, liberated FA’s can enter the beta oxidation cycle to produce acetyl-CoA and the rate limiting enzyme for this process is the β-hydrocyacyl-CoA dehydrogenase (β-HAD). The maximal activity of this rate limiting enzyme was shown to be elevated after 2-wk of fat adaptation (Goedecke, et al., 1999; Table 1.2). However, Fisher et al. (Fisher, et al., 1983) demonstrated that hexokinase activity was reduced by 4 wk fat adaptation (Table 1.2), suggesting that apart from increased fat oxidation ability, the ability to oxidise glucose may actually be compromised by fat adaptation strategies.

Perhaps most importantly, fat adaptation strategies result in reduced muscle glycogen content, and regardless of the level to which fat oxidation is elevated during exercise, it is of utmost important for athletes to approach endurance sporting competitions with maximised glycogen storage (Burke, et al., 2004) as muscle glycogen content has long been associated with fatigue during exercise (Bergstrom, et al., 1967).
1.3.2 Fat adaptation and CHO restoration

Background and protocols

To circumvent the issue of reduced glycogen storage as a result of high-fat diets, several groups (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001; Lambert, et al., 2001; Rowlands and Hopkins, 2002; Stellingwerff, et al., 2006) have proposed that a ‘dietary periodisation’ model involving fat adaptation with CHO restoration would result in optimising both rates of fat oxidation while concomitantly maximising glycogen storage. In this model, a period of fat adaptation (~5-12 days, ~4.6 g/kg/day or ~70% energy from fat and ~15% energy from CHO) is followed by a short term (1-3 days, ~15% energy from fat and ~10.3 g/kg/day or ~70% energy from CHO) CHO restoration phase (Figure 1.1). It was hypothesised that the short restoration phase (1-3 days) would be sufficient to restore glycogen, but at least part of the elevated fat oxidative response to fat adaptation would persist even in the face of higher glycogen content (Burke, et al., 2000). This was critical to the concept, since after a ‘standard’ high CHO diet, high starting muscle glycogen concentration coincides with a correspondingly high utilisation rate during exercise. Consequently physiological adaptations that allow an increased rate of fat oxidation and sparing of glycogen in spite of high starting glycogen content should in theory, represent the ideal scenario for maximizing endurance capacity.

In general, protocols that have utilised fat adaptation with CHO restoration have utilised a cross-over design, whereby a “control diet” consisting of a high CHO diet (containing the same macronutrient and fibre composition as CHO restoration) is compared to the fat adaptation and CHO restoration diet (Figure 1.1). Subjects are also required to maintain their regular training programme, including high intensity training sessions, throughout the duration of the study.
Interestingly, even though studies have used from 1-3 days to restore muscle (and liver) glycogen stores, it has been shown that only 1 day of CHO restoration is necessary to “top up” muscle glycogen concentration in endurance-trained athletes (Burke, et al., 2000; Stellingwerff, et al., 2006). A consistent finding is that, while CHO restoration suppressed fat oxidation relative to the levels seen prior to restoration, rates of fat oxidation remain higher than prior to initiation of the fat adaptation phase (Burke, et al., 2000; Stellingwerff, et al., 2006). Even when CHO availability is increased acutely (by feeding a high CHO breakfast prior to, or ingesting CHO during exercise), elevated rates of fat oxidation persist (Burke, et al., 2002; Carey, et al., 2001). It was also shown that the reduction in CHO oxidation observed during exercise after fat adaptation and CHO restoration strategies can be accounted for by the reduction in muscle glycogen utilisation during exercise, despite the fact that muscle glycogen was restored to a high level after the CHO restoration phase (Burke, et al., 2000; Lambert, et al., 2001; Table 1.2).
Table 1.2  
Summary of studies investigating fat adaptation with and without carbohydrate restoration in well-trained athletes

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Protocol</th>
<th>FAT&lt;sub&gt;ox&lt;/sub&gt; (vs. Cont)</th>
<th>Skeletal muscle adaptation (vs. Cont)</th>
<th>Performance (vs. Cont)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phinney et. al.</td>
<td>n=5 male Well-trained cyclists (&gt; 65 mL/kg/min)</td>
<td>One-week eucaloric balanced diet followed by 4-week ketogenic diet</td>
<td>↑ 3 fold ↓ in glucose oxidation; 4 fold ↓ in glycogen utilization</td>
<td>Time to exhaustion ↔</td>
<td></td>
</tr>
<tr>
<td>Fisher et. al.</td>
<td>n=5 male Well-trained cyclists (5.1 L/min)</td>
<td>One-week eucaloric balanced diet followed by 4-week ketogenic diet</td>
<td>↑ ↓ glycogen content; ↓ glycogen utilization ↑ CPT &amp; ↓ hexokinase activities</td>
<td>Time to exhaustion ↔ (60% VO&lt;sub&gt;2max&lt;/sub&gt;)</td>
<td></td>
</tr>
<tr>
<td>Muoio et. al.</td>
<td>n=6 male Well-trained runners (63.7 mL/kg/min)</td>
<td>Cont: Normal Expt: HCHO Expt: HFAT</td>
<td>↔</td>
<td>HFAT ↑ VO&lt;sub&gt;2max&lt;/sub&gt; and running time to exhaustion</td>
<td></td>
</tr>
<tr>
<td>Lambert et. al.</td>
<td>n=5 male Well-trained cyclists (4.2 L/min)</td>
<td>Cont: 2 weeks HCHO Expt: 2 weeks HFAT</td>
<td>↑ ↓ glycogen content (resting)</td>
<td>HFAT ↑ time to exhaustion (60% VO&lt;sub&gt;2max&lt;/sub&gt;)</td>
<td></td>
</tr>
<tr>
<td>Goedeeke et. al.</td>
<td>n=16 male Well-trained cyclists (63.5 mL/kg/min)</td>
<td>Cont: Habitual Expt: HFAT</td>
<td>↑ ↓ glycolysis oxidation; ↑ CPT activities; citrate synthase β-HAD ↔</td>
<td>40km time trial (TT) ↔</td>
<td></td>
</tr>
<tr>
<td>Burke et. al.</td>
<td>n=8 male Well-trained cyclists (64.4 mL/kg/min)</td>
<td>Cont: 6-day HCHO Expt: 5-day HFAT + 1-day HCHO</td>
<td>↑ ↓ glycogen content after 5-day HFAT but restored after 1-day HCHO ↓ glycogen utilization</td>
<td>TT performance (7kj/kg) with 120 min preload ↔</td>
<td></td>
</tr>
<tr>
<td>Carey et. al.</td>
<td>n=7 male Well-trained cyclists (5.06 L/min)</td>
<td>Cont: 7-day HCHO Expt: 6-day HFAT + 1-day HCHO</td>
<td>↑</td>
<td>60 min performance trial (km) with 4 hr preload ↔</td>
<td></td>
</tr>
<tr>
<td>Lambert et. al.</td>
<td>n=5 male Well-trained cyclists (4.9 L/min)</td>
<td>Cont: 10-day habitual diet + 3-day HCHO Expt: 10-day HFAT + 3-day HCHO</td>
<td>↑ ↓ estimated rates of muscle glycogen &amp; lactate oxidation</td>
<td>↑ 20-km TT performance (with 150 min preload)</td>
<td></td>
</tr>
<tr>
<td>Rowlands &amp; Hopkins</td>
<td>n=7 male Well-trained male (72 mL/kg/min)</td>
<td>Cont: 14-day HCHO Expt: 14-day HFAT Expt: 11.5-day HFAT + 2.5-day HCHO</td>
<td>↑</td>
<td>HFAT &amp; HFAT + CHO ↓ decline in power output during 100km TT</td>
<td></td>
</tr>
<tr>
<td>Burke et. al.</td>
<td>n=8 male Well-trained cyclists (68.6 mL/kg/min)</td>
<td>Cont: 6-day HCHO Expt: 5-day HFAT + 1-day HCHO</td>
<td>↑</td>
<td>Plasma glucose uptake ↔ TT performance (7kj/kg) with 120 min preload ↔</td>
<td></td>
</tr>
<tr>
<td>Cameron-Smith et. al. 2003</td>
<td>n=14 male Well-trained male (67 mL/kg/min)</td>
<td>Cont: 5-day HCHO Expt: 5-day HFAT</td>
<td>↑</td>
<td>HFAT ↑ FAT/CD36 (protein &amp; mRNA) &amp; β-HAD mRNA</td>
<td></td>
</tr>
<tr>
<td>Havemann et. al.</td>
<td>n=8 male Well-trained (57.8 mL/kg/min)</td>
<td>Cont: 7-day HCHO Expt: 6-day HFAT + 1-day HCHO</td>
<td>↑</td>
<td>Normalized EMG amplitude during 1-km sprint ↔ TT performance (100km) ↔</td>
<td></td>
</tr>
<tr>
<td>Stellingwerff et. al. 2006</td>
<td>n=7 male Well-trained cyclists (60.7 mL/kg/min)</td>
<td>Cont: 6-day HCHO Expt: 5-day HFAT + 1-day HCHO</td>
<td>↑ ↓ PDH activity; ↓ glycolgenolysis ↓ estimated substrate phosphorylation</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

↑, increase; ↓, decrease; ↔, unchanged; Cont, Control group; Expt, Experimental group; HCHO, High carbohydrate diet; HFAT, High fat diet.
**Fat adaptation and carbohydrate restoration: potential mechanisms of action**

A recent study by Stellingwerff et al. (2006) has provided a new insight into the mechanisms by which fat adaptation with or without CHO restoration modifies substrate metabolism during exercise. Utilising a five day fat adaptation followed by one day CHO restoration protocol and well-trained athletes as subjects, Stellingwerff et al., (2006) reported that pyruvate dehydrogenase (PDH) activity was 56% lower at rest and 29% lower after a 20-min submaximal cycle exercise after fat adaptation and CHO restoration intervention. Moreover, a reduced PDH activity was also demonstrated after a 1-min sprint cycle exercise following this dietary intervention (Stellingwerff, et al., 2006). Calculated rates of muscle glycogenolysis were significantly lower during the first minute of both the submaximal and sprint cycle exercise (9.1 ± 1.1 vs. 13.4 ± 2.1 and 37.3 ± 5.1 vs. 50.5 ± 2.7 glucosyl units/kg/min dry wt. respectively) and estimated substrate phosphorylation was also lower during the first minute of the submaximal cycle exercise after fat adaptation and CHO restoration trial (Stellingwerff, et al., 2006). Collectively, these results indicated that the increased fat oxidation capacity observed after fat adaptation and CHO restoration may well be an artifact of a decrease in the ability to oxidise CHO (i.e. an impairment to CHO breakdown).

While the results of Stellingwerff et al. (2006) are intriguing, it is important to note that the actual metabolic signals that accounted for the shifts in substrate selection during exercise and associated skeletal muscle adaptations after fat adaptation and CHO restoration remain largely unknown. In this regard, the proposed role of AMPK as the ‘fuel sensor’ in skeletal muscle suggests that this ubiquitous protein may play a role in the altered muscle metabolism that accompanies fat-adaptation strategies. Figure 1.2A showed the intramyocellular and sarcolemmal
environment in endurance trained humans before fat adaptation strategies. Endurance training has been known to increase skeletal muscle FA transporter protein, FAT/CD36 (Bonen, et al., 1999; Koonen, et al., 2004; Simoneau, et al., 1999) and glucose transporter protein, GLUT4 (Burgomaster, et al., 2007; Daugaard, et al., 2000; Gjovaag and Dahl, 2009). Endurance training also increases AMPK and PDH activity, mitochondrial volume density and enzymes such as β-HAD and CPT1, along with increases in both muscle glycogen (Hargreaves, 1997; Hawley, et al., 1997) and TG concentration (Hoppeler, et al., 1973).

A high fat diet in conjunction with endurance training during fat adaptation has been reported to alter the muscle substrate profile where muscle TG is increased while muscle glycogen is decreased (Figure 1.2B). This high-lipid, low-CHO environment may activate AMPK and increase the rates of fat oxidation observed through the AMPK-ACCβ-CPT1 signalling axis (to be discussed subsequently) (Figure 1.2B). In addition, AMPK may also be activated by the interactive effects of diet and exercise. Lessard et al. (2007) recently reported that AMPKα1 activity was only elevated in high-fat fed rodents when they concurrently undertook an intense endurance training programme, further suggesting the potential of AMPK as the regulator of the metabolic adaptation seen in this model of nutrient-training intervention. The increases in β-HAD activity and FAT/CD36 observed after fat adaptation strategies could also be due to the activation of AMPK as it has been previously reported that AMPK activation led to an increased in β-HAD activity in skeletal muscle (Putman, et al., 2003), and FAT/CD36 protein content in both skeletal muscle (Bonen, et al., 2007) and cardiac myocytes (Chabowski, et al., 2006) (Figure 1.2B).
The provision of a high-CHO diet and rest during the CHO restoration phase has been demonstrated to restore muscle glycogen content to the level seen prior to fat adaptation intervention (Burke, et al., 2000; Stellingwerff, et al., 2006) (Figure 1.2C). However, it is currently not known if the increased muscle TG content observed after fat adaptation protocol would remain after this CHO restoration phase (Figure 1.2C). Should this be the case, the increased lipid availability may also activate AMPK to maintain the adaptations associated with fat adaptation protocol previously discussed, although this may happen to a lesser degree as the restoration of muscle glycogen may provide certain level of inhibition towards AMPK activation (McBride, et al., 2009) (Figure 1.2C). Taken collectively, it is likely that the adaptations seen previously with fat adaptation strategies are mediated in part through AMPK.
Figure 1.2  Endurance training and fat adaptation induced skeletal muscle adaptation with and without carbohydrate restoration. A) Intramyocellular and sarcolemmal environment in an endurance trained human before dietary manipulation, B) Fat adaptation-induced perturbations in skeletal muscle fuel availability, adaptations and associated putative signalling pathways C) Fat-adaptation followed by carbohydrate restoration-induced perturbations in skeletal muscle fuel availability, adaptations and associated putative signalling pathways. Solid lines denote signalling that has been verified experimentally after fat adaptation studies, whereas dashed lines signify putative steps that are yet to be validated. FAT/CD36, Fatty acid translocase; Gly, Glycogen; TG, triglyceride; PDH, Pyruvate dehydrogenase; ATGL, Adipose triglyceride lipase; HSL, Hormone sensitive lipase; αβγ, 5’ AMP-activated protein kinase; ACC, Acetyl-CoA-carboxylase; β-HAD, β-hydroxyacyl-CoA-dehydrogenase; CPT1, carnitine palmitoyltransferase 1; P, Phosphorylation.

1.3.3 Training with low muscle glycogen availability

While the role of muscle glycogen in endurance performance has been well established, its role in skeletal muscle training adaptation is less certain. Indeed, it is not currently known whether it is a lack or a surplus of glycogen that triggers skeletal muscle to adapt in response to exercise training. In recent years, the role of glycogen has evolved from being merely a fuel store to that of a regulator of cellular signalling and function (Hargreaves, 2004; Hawley, et al., 2006). In this regard, several signalling proteins and transcription factors has been shown to possess a glycogen binding domain (McBride, et al., 2009; Polekhina, et al., 2003). Indeed, in recent years it has become evident that commencing exercise in the face of low muscle glycogen stores amplifies
the activation of a number of signalling proteins, including the AMPK (Steinberg, et al., 2006; Wojtaszewski, et al., 2003) and the p38 MAPK (Chan, et al., 2004), two enzymes with direct roles in controlling the expression and activity of several transcription factors involved in mitochondrial biogenesis and promoting training adaptation [for review, see (Baar and McGee, 2008; Hawley, et al., 2006)].

While the effects of acute exercise in the face of low muscle glycogen on skeletal muscle adaptive responses has been investigated (Chan, et al., 2004; Steinberg, et al., 2006; Wojtaszewski, et al., 2003), the chronic effects of training in the face of low muscle glycogen stores has received less attention. Only one study to date has examined the effects of a training programme in which a portion of exercise sessions were deliberately commenced with low muscle glycogen on subsequent skeletal muscle adaptation and performance (Hansen, et al., 2005). Using an ingenious design, Hansen et al. (2005) recruited seven untrained males to undertake a 10-wk programme of leg knee extensor “kicking” exercise in which both of the subject’s legs were trained according to a different daily schedule, but the total amount of work undertaken by each leg over the study duration was the same: subjects trained one leg twice a day, every second day, whereas the contralateral (control) leg was trained daily. Interestingly, compared with the leg that performed daily training with normal glycogen reserves, the leg that commenced half of the training sessions with low muscle glycogen levels had a more pronounced increase in resting glycogen content and citrate synthase activity (Hansen, et al., 2005). Remarkably, this “train-low” approach resulted in an almost twofold greater training-induced increase in one-leg exercise time to fatigue compared with when participants commenced all training sessions in a glycogen-replete state.
While the results of that study (Hansen, et al., 2005) are intriguing, it was hard to discern the practical significance of the findings. First, the intensity of the training sessions in the Hansen et al. (2005) study was ‘clamped’ at a submaximal intensity for both legs. Training with high muscle glycogen concentration may enable an individual to train at a higher relative intensity and therefore achieve a more superior adaptation. Second, the mode of training (one-legged knee extensor kicking) and the exercise performance test utilised (submaximal kicking to exhaustion) provide little resemblance to normal training modes (whole-body exercise) and performance tasks in real world competitive races. Third, training schedules that induce chronically low muscle glycogen concentration may result in over-reaching or overtraining and thereby affect training adaptation and performance (Petibois, et al., 2003). Clearly, there appears to be a need to re-visit this research question in a model utilising whole-body exercise and where performance is assessed using exercise test that mimics real life sporting situations. In addition, the subjects should be allowed to freely choose their maximal effort during training rather than ‘clamping’ their training intensity to account for differences in ability to perform training with different levels of muscle fuel stores. Moreover, should this training strategy be used, it should be viewed in the overall context of an athlete’s training programme, and ‘periodised’ in accordance with the goals of the athlete in mind.

1.3.4 Fed versus fasted training

While performing acute exercise in the face of low muscle glycogen has been shown to increase the activation of AMPK and p38 MAPK (Chan, et al., 2004; Steinberg, et al., 2006; Wojtaszewski, et al., 2003), exercise with oral CHO ingestion has resulted in equivocal responses. Lee-Young et al. (2006) reported no effects of CHO ingestion on AMPK signalling during
exercise, whereas Akerstrom et al. (2006) reported oral glucose ingestion attenuated the exercise-induced activation of AMPK. However, using young healthy humans as subjects, De Bock et al. (2005) showed that muscle TG breakdown during an acute bout of prolonged submaximal exercise in the fasted state was increased while this breakdown is attenuated in the CHO-fed state. In addition, glycogen resynthesis after exercise was also enhanced in fasted state compared to the CHO-fed state (De Bock, et al., 2005).

These observations have prompted two groups of researchers to investigate the effects of ‘fed’ versus ‘fasted’ training on skeletal muscle adaptation and performance. In one study, De Bock et al. (2008) recruited 20 moderately active male who undertook a 6-wk training programme (3 day/wk, 1–2 h, 75% VO$_{2\text{peak}}$). One group (n=10) performed all the training sessions in a fed state while the other group (n=10) performed the training sessions in fasted state. The authors (De Bock, et al., 2008) reported that the abundance of the fatty acid transporter protein, plasma membrane fatty acid binding protein (FABP$_{pm}$) was increased in the group that performed training in the fasted state (De Bock, et al., 2008). In addition, for a given initial glycogen content, exercise-induced glycogen breakdown was blunted in the fasted training group when compared to the fed training group. However, neither muscle TG breakdown nor rates of fat oxidation was altered by these divergent training programmes. Moreover, peak VO$_2$, succinate dehydrogenase activity, GLUT4, and hexokinase II content were increased to a similar extent between the two groups (De Bock, et al., 2008).

In a recent study, Akerstrom et al. (2009) recruited nine physically active, healthy, male subjects to investigate the effects of a 10-wk one-legged knee extensor training programme with or
without glucose ingestion on subsequent skeletal muscle adaptation and performance. The subjects trained one leg while ingesting a 6% glucose solution and ingested a sweetened placebo while training the other leg. The subjects trained their respective legs 2 hr at a time on alternate days, 5 days a week for 10-wk. Peak power, time to fatigue at 70% maximal power output, glycogen content and the maximal activities of CS and β-HAD were increased to a similar extent, while the rate of appearance and disappearance for both palmitate and glucose were not different when subjects trained with or without glucose supplementation (Akerstrom, et al., 2009). It was concluded that glucose ingestion during training does not alter training adaptation related to substrate metabolism, mitochondrial enzyme activity, glycogen content or performance (Akerstrom, et al., 2009).

In summary, it is evident that several of the nutrient-training strategies reviewed are capable of modulating skeletal muscle metabolism and the adaptation to a training stimulus. However, there is a need to further investigate the interactive effects of nutrient-training interactions on selected metabolic and signalling pathways such that a better understanding of these two potent stimuli interact to modify muscle adaptation.
1.4 Signalling proteins regulated by both exercise training and nutrient availability

Over the past few decades, significant progress has been made whereby certain molecular biology techniques such as real-time polymerase chain reaction (RT-PCR), and high-throughput chip assays are now available to investigate the effects of various training interventions on a variety of early-response genes and signalling proteins in skeletal muscle. Such advances have permitted not only the identification of the cellular and molecular factors that are triggered by the perturbations in metabolic homeostasis in response to exercise, but also the signalling mechanisms by which the activation of these cellular and molecular factors are translated into subsequent skeletal muscle adaptation. As chronic adaptation to training is believed to be the cumulative effects of repeated bouts of acute exercise (Hansen, et al., 2005; Hawley, et al., 2006; Mahoney and Tarnopolsky, 2005), such acute metabolic and signalling responses to exercise may provide important clues of how training and nutrition interventions may modulate subsequent training adaptations.

In this regard, acute exercise is known to result in rapid increases in cytosolic and mitochondrial calcium ($\text{Ca}^{2+}$) and depending on the relative intensity and duration of the exercise, increases in AMP/ATP and PCr/[PCr + creatine (Cr)] ratio, and also muscle lactate. Again, depending on the relative intensity and duration, exercise is also known to result in a decrease in muscle substrate, such as glycogen (Coyle, et al., 1986) and triglyceride (TG) (Helge, et al., 2006; Hurley, et al., 1986; Stellingwerff, et al., 2007b). Together, these perturbations in metabolic homeostasis, coupled with exercise associated mechanical stress result in the activation of several protein kinases that have been shown to have a role in mediating exercise-induced mitochondrial biogenesis, such as the AMPK and the p38 mitogen-activated protein kinase (MAPK). Of
particular interest is that two of the major proteins that are phosphorylated by exercise (the AMPK and p38 MAPK) have also been shown to be activated by the prevailing nutrient availability (Steinberg, et al., 2006; Watt, et al., 2006; Wojtaszewski, et al., 2003). Accordingly, it may be that these two signalling proteins provide a mechanistic link between nutrient availability and skeletal muscle adaptation in response to endurance training.

1.4.1 5’ AMP activated protein kinase

AMPK is a heterotrimeric enzyme consisting of one α catalytic subunit and two regulatory, β and γ subunits. The α- and β- subunits each exist in two isoforms (α1 and α2, β1 and β2) while the γ-subunit exists in three isoforms (γ1, γ2 and γ3). The AMPK α-subunit contains the kinase domain and the phosphorylation site threonine 172 which is activated by upstream kinases (Hawley, et al., 1996). The β-subunit contains a glycogen binding domain (McBride, et al., 2009; Polekhina, et al., 2003) while the γ-subunit contains two pairs of Bateman [CBS (cystathionine β-synthase)] domains that act as the binding site for AMP and ATP (Figure 1.3).

The binding of AMP molecules to the Bateman domain activates AMPK while ATP competes with AMP for binding to these domain sites and is an inhibitor of AMPK (Scott, et al., 2004; Xiao, et al., 2007). During resting conditions where cells energy status is not depressed, ATP is believed to be predominantly bound to the Bateman domains and AMPK molecules are inactive (Xiao, et al., 2007) (Figure 1.4). However, during muscular contraction (i.e. exercise), ATP is hydrolysed into adenosine diphosphate (ADP) before being converted rapidly into AMP by the adenylate kinase reaction. Depending on the intensity and duration of the contraction (i.e. exercise), this in turn leads to an accumulation of AMP and consequently AMPK is activated.
AMPK can also be phosphorylated by upstream kinases such as LKB1 and calcium/calmodulin-dependant kinase kinase (CaMKK) in response to changes in cellular concentrations of AMP and calcium (Ca\(^{2+}\)).

**α-subunit**

**β-subunit**

**γ-subunit**

Figure 1.3  Typical domain structure of the α, β and γ subunits of AMPK [reproduced from Hardie, et al. (2006)].
In line with its proposed role as the ‘metabolic master switch’, AMPK is activated by conditions resulting in cellular metabolic stress by either an increase in the rate of ATP hydrolysis, such as during exercise, or during events where ATP production is inhibited, such as during ischaemia and hypoxia. This activation of AMPK subsequently results in the activation of cellular processes that increase energy production [e.g., increased glucose uptake and/or fat oxidation (Merrill, et al., 1997)] while concomitantly inhibiting processes that require energy consumption (e.g., protein synthesis) to restore energy levels. In this regard, AMPK is believed to regulate fatty acid oxidation by phosphorylating and inactivating its downstream target acetyl-CoA-carboxylase-β (ACCβ). The decrease in the activity of ACCβ in turn leads to the lowering of malonyl CoA levels and subsequently relieving malonyl CoA inhibition on carnitine palmitoyltransferase 1 (CPT1). Since CPT1 is the rate limiting enzyme that controls the long-chain FA entry into the
mitochondrial matrix, the activation of AMPK and subsequent activation and inhibition of ACCβ resulted in an increase in FA oxidation (Winder and Hardie, 1996).

The role of AMPK in mediating exercise-induced mitochondrial biogenesis has been demonstrated in animal studies where pharmacological agents have been used to activate AMPK. In their seminal studies, Winder and colleagues (Holmes, et al., 1999; Winder, et al., 2000) showed that chronic activation of AMPK by aminoimidazole carboxamide ribonucleotide (AICAR) injections increased the protein expression of hexokinase, GLUT4, and several mitochondrial enzymes in rat skeletal muscle, an effect similar to that of endurance training. In a later study, Zong et al. (2002) fed mice with β-guanidinopropionic acid (GPA), a creatine analogue that leads to increases in intramuscular AMP/ATP ratio. The authors (Zong, et al., 2002) reported that AMPK activity and subsequent mitochondrial biogenesis was only observed in the control mice but not in the experimental mice overexpressing a dominant-negative AMPK in muscle. Together, these results indicated that AMPK activation increases mitochondrial biogenesis and may mediate some of the mitochondrial adaptations that take place in response to exercise training.

In human skeletal muscle, AMPK can be activated during exercise in a rapid, intensity-dependant and isoform specific manner. Chen et al. (2000) demonstrated that both AMPKα1 and -α2 isoforms are rapidly activated during a 30-s maximal sprint cycle exercise. With respect to exercise intensity, Fujii et al. (2000) reported that AMPK activity was significantly increased in the vastus lateralis muscle following 20 min of cycling at 70% but not at 50% VO₂peak while AMPK has also been demonstrated to be activated by four 30-s "all out" sprint cycle bouts
AMPK isoform specific activation has also been reported by Chen et al. (2003). Specifically, the activation status of both AMPKα1 and –α2 activity was increased when exercise intensity was increased from ~40% VO_{2peak} to ~60% VO_{2peak} but when the exercise intensity was further increased to ~80% VO_{2peak}, only AMPKα2 but not α1 activity was increased. With regards to exercise duration, AMPK activity was found to be increased during the first 5 min of moderate (60% of VO_{2peak}) cycling with further rises observed after 30 min of exercise (Stephens, et al., 2002). AMPK has also been shown to become progressively more phosphorylated during prolonged (~210 min) low intensity (45% VO_{2peak}) cycling exercise (Wojtaszewski, et al., 2002).

The activation of AMPK by exercise is reduced by endurance training (McConell, et al., 2005; Nielsen, et al., 2003; Yu, et al., 2003). Yu et al. (2003) demonstrated that the AMPK activation in response to a single bout of exercise (undertaken at the same relative intensity) was reduced in well-trained endurance athletes compared to untrained sedentary subjects matched for age and body mass. In agreement with Yu et al. (2003), Nielsen et al. (2003) reported that phosphorylation states of AMPKα at theonine 172 was significantly blunted in seven well-trained endurance athletes compared to seven young, healthy sedentary men. In another study, McConell et al. (2005) showed that AMPK activation in response to exercise was attenuated when untrained subjects went through a short-term (10 days) endurance training programme. Indeed, it appears that intense (85% VO_{2peak}) exercise is required to activate AMPK in well-trained individuals (Clark, et al., 2004).
AMPK expression has also been shown to be regulated by endurance training in both cross-sectional and longitudinal training studies. Comparing seven well-trained endurance athletes to seven young, healthy sedentary men, Nielsen et al. (2003) reported that the protein content of AMPKα1 isoform was significantly higher in the well-trained endurance athletes. Frosig et al. (2004) recruited eight healthy young men to undertake supervised one-legged knee extensor endurance training for 3 weeks. In response to the training programme, the protein content of AMPK-α1, -β2 and -γ1 isoform was increased in the trained leg by 41, 34 and 26% respectively, whereas the regulatory γ3 isoform was decreased by 62%. However, no effect was observed on the remaining α2, β1 and γ2 isoform. More importantly was the observation that the activity of the catalytic subunit α1 and α2 at rest was elevated by 94 and 49% respectively, indicating that AMPK activity is upregulated by endurance training.

While the AMPK activity has been shown to be upregulated by training, it is interesting to note that the AMPK activation can also be regulated by substrate availability in human skeletal muscle. Wojtaszewski et al. (2003) reported that compared to a glycogen loaded state (900 mmol/kg dry wt), the resting activities of the AMPK-α1 and -α2 isoform were significantly higher by 160 and 145% respectively when muscle glycogen content was reduced (~160 mmol/kg dry wt.) while the activity of the AMPKα2 isoform was also found to be higher when exercise (1 hour cycle at 70% VO2peak) was performed in the face of low glycogen availability (Wojtaszewski, et al., 2003). In agreement with Wojtaszewski et al. (2003), Steinberg et al. (2006) also reported that when seven males performed 60-min of exercise on two occasions, commencing exercise with low compared to normal glycogen levels was associated with elevated AMPKα2 activity and increased AMPKα2 translocation to the nucleus. In support of this contention, AMPK has been shown to
possess a glycogen binding domain within its β isoform (McBride, et al., 2009; Polekhina, et al., 2003) and recently, McBride et al. (2009) provided the first evidence that AMPK activity is indeed inhibited by glycogen.

However, it is important to note that the glycogen depleted state in Wojtaszewski et al. (2003) also resulted in a two fold increase in plasma long chain FA’s and the dietary intervention used to create the glycogen depleted state in both the studies of (Steinberg, et al., 2006) and (Wojtaszewski, et al., 2003) may also have resulted in increased muscle TG stores. Watt et al. (2006) reported that the incubation of L6 skeletal muscle myotubes with palmitate and linoleate increased AMPK activity significantly and that this increased in AMPK activity occurred in the absence of detectable changes in AMP and glycogen content, indicating that fatty acids may have a modulatory role on AMPK activity (Watt, et al., 2006). While most studies have proposed a role for muscle glycogen availability in modifying metabolic signals (i.e. AMPK), the role of the diet-induced increase in fat availability should not be overlooked.

1.4.2 p38 mitogen-activated protein kinase

p38 MAPK is a member of the mitogen-activated protein kinase family that consists of p38 MAPK, extracellular signal-regulated kinases (ERK) 1 and 2 (ERK1/2), c-Jun NH₂-terminal kinases (JNK) and ERK5 (Kramer and Goodyear, 2007). p38 MAPK is activated by various stimuli such as muscular contraction, insulin, environmental stress and proinflammatory cytokines (Raingeaud, et al., 1995; Somwar, et al., 2000; Tsakiridis, et al., 1996). p38 MAPK has been implicated to be involved in a variety of diverse roles including skeletal muscle myogenic
cell differentiation (Zetser, et al., 1999), regulation of muscle atrophy and mitochondrial biogenesis.

The activation of p38 MAPK during exercise has been observed during treadmill exercise in rodents (Goodyear, et al., 1996; Nader and Esser, 2001), cycling ergometry (Benziane, et al., 2008; Widegren, et al., 1998), marathon running (Boppart, et al., 2000; Yu, et al., 2001) and sprint cycling (Gibala, et al., 2009) in humans. The role of p38 MAPK in mediating mitochondrial biogenesis in response to exercise has been demonstrated in a series of experiments utilising animals, cell and transgenic models (Akimoto, et al., 2005). These authors (Akimoto, et al., 2005) initially showed that voluntary running exercise concurrently increased p38 MAPK and peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), a transcriptional co-activator that promotes mitochondrial biogenesis (to be discussed subsequently) in mouse skeletal muscle (Akimoto, et al., 2005). Using C2C12 cultured myocytes, they then went on to show that the activation of p38 MAPK stimulated PGC-1α promoter activity and that this increased in PGC-1α promoter activity can be blocked by specific p38 inhibitors, or dominant negative p38 (Akimoto, et al., 2005). On a more functional note, they showed that constitutively over-expressed skeletal muscle-specific p38 in transgenic mice resulted in enhanced PGC-1α and cytochrome oxidase IV protein expression in fast-twitch skeletal muscles (Akimoto, et al., 2005). Taken collectively, these results suggested that p38 MAPK plays a role in mediating mitochondrial biogenesis in response to exercise.

Interestingly, the p38 MAPK has also been shown to be regulated by substrate availability in human skeletal muscle (Chan, et al., 2004). Chan et al. (2004) had eight subjects performed 60
min of exercise on two occasions; either with prior ingestion of a normal (Con) or low carbohydrate (LCHO) diet that reduced pre-exercise muscle glycogen content. Chan et al. (2004) found that the phosphorylation of nuclear p38 was greater both at rest and after exercise in the group that performed the exercise with low muscle glycogen content. Again, similar to studies that reported regulation of AMPK by muscle glycogen availability (Steinberg, et al., 2006; Wojtaszewski, et al., 2003), Chan et al. (2004) may have overlooked that their dietary intervention may also have altered muscle lipid profile and that these changes in lipid profile may have a role on the increased p38 MAPK activation. Nevertheless, the important message is the p38 MAPK appears to be regulated by muscle substrate availability and in view of the role of the p38 MAPK in mediating mitochondrial biogenesis in response to exercise, p38 MAPK may serve as one of the candidate signalling proteins that can potentially mediate the interactive effects of nutrient availability and exercise training.

1.4.3 Downstream signalling pathways of AMPK and p38 MAPK

Peroxisome proliferator-activated receptor gamma coactivator-1α

The AMPK and p38 MAPK are believed to mediate mitochondrial biogenesis in response to exercise through a similar downstream target, PGC-1α. PGC-1α is a member of the family of transcriptional regulators consisting of PGC-1α, peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-β (PGC-1β) and PGC-1 related co-activator (PRC). Being a transcriptional co-activator, PGC-1α does not bind to DNA itself in a sequence-specific manner (Liang and Ward, 2006; Puigserver and Spiegelman, 2003). Instead, it recruits histone acetyl transferase (HAT) enzymes to a number of transcription factors such as the myocyte enhancer factor 2 (MEF2), estrogen related receptor α (ERRα), nuclear respiratory factor-1 (NRF-1) and -2
(NRF-2) and subsequently modifies the chromosome structure to one that favours transcriptional activation to regulate glucose and FA metabolism, mitochondrial biogenesis, and muscle fiber type transformation (Czubryt, et al., 2003; Handschin, et al., 2003; Lin, et al., 2002; Puigserver, et al., 1998; Vega, et al., 2000; Wu, et al., 1999).

PGC-1α signalling can be increased by either increasing its activity or protein expression. Wright et al. (2007) provided first evidence that the increases in mitochondrial enzyme expression occurred before the increased in PGC-1α protein expression and concluded that exercise rapidly increases PGC-1α activity to mediate the initial phase of the exercise-induced adaptive increase in muscle mitochondria content (Wright, et al., 2007). On the other hand, the increased in PGC-1α protein has been shown to be the result of increased transcription of PGC-1α gene (Akimoto, et al., 2004). This increased in the transcription of PGC-1α gene is regulated by proteins binding to either the MEF2 or cyclic AMP response elements (CRE) within the PGC-1α promoter (Akimoto, et al., 2008; Akimoto, et al., 2004).

Both PGC1-α mRNA and protein expression have been reported to increase in response to exercise. In both mice and humans, PGC-1α mRNA was reported to be increased by ~1.5–10 fold after a single bout of exercise (Baar, et al., 2002; Gibala, et al., 2009; Mathai, et al., 2008; Pilegaard, et al., 2003; Terada, et al., 2002; Terada, et al., 2005; Wright, et al., 2007). However, the increased in PGC-1α protein expression after exercise has not been consistently demonstrated in human skeletal muscle. Mathai et al (2008) reported that skeletal muscle PGC-1α protein was increased in response to an acute bout of exercise whereas it was unchanged after exercise in another study (Gibala, et al., 2009). Nevertheless, the activation of PGC-1α has also been shown
in cell culture models by stimulus that mimic aspects of endurance exercise (Akimoto, et al., 2005; Irrcher, et al., 2003; Lee, et al., 2006; Ojuka, et al., 2003). Terada et al. (2005) further showed that PGC-1α activation during exercise is intensity dependant with greater activation observed after high intensity intermittent compared to low intensity prolonged swimming exercise (Terada, et al., 2005). Collectively, these results demonstrate that PGC-1α is activated by exercise.

PGC-1α has been postulated to be a key regulator of mitochondrial biogenesis. Over-expressing PGC-1α in mice results in greater expression of skeletal muscle mitochondrial enzymes and oxidative capacity while electrically stimulating muscles from these transgenic mice is associated with a 3-fold increase in time to fatigue compared to their wild-type littermate (Lin, et al., 2002). However, as over-expression of PGC-1α often resulted in a 10-30 fold increased in PGC-1α expression, it has been questioned whether such large perturbations can be achieved with physiological stimuli (i.e. exercise training). To address this question, Benton et al. (2008) used an electrotransfection procedure to over-express PGC-1α rapidly (2 weeks), within physiologic limits, in rat tibialis anterior muscle in one limb while the contralateral muscle served as control. They (Benton, et al., 2008) reported a modest (24%) increase in PGC1-α protein expression such that might be expected in response to exercise (Mathai, et al., 2008), or after endurance training (Burgomaster, et al., 2008) in human skeletal muscle. This modest increased in PGC1-α was sufficient to induce mitochondrial biogenesis as shown in an increased in citrate synthase activity, cytochrome c oxidase subunit 4 (COX IV) and mitochondrial DNA while concomitantly increased FA oxidation in subsarcolemmal mitochondria (Benton, et al., 2008). Together, these results suggested that PGC1-α is indeed a powerful regulator of mitochondrial biogenesis.
The AMPK and p38 MAPK signalling pathways are believed to converge at PGC-1α to mediate mitochondrial biogenesis (Figure 1.6). There are several mechanisms proposed by which AMPK interacts with PGC-1α to induce mitochondrial adaptations. Recently, using primary muscle cells, Jager et al. (2007) showed that the effects of AMPK on gene expression of glucose transporter 4 (GLUT4), mitochondrial genes, and PGC-1α itself are almost entirely dependent on the function of PGC-1α protein. In addition, these workers (Jager, et al., 2007) demonstrated that AMPK phosphorylated PGC-1α directly both in vitro and in cells and these direct phosphorylations of the PGC-1α protein at threonine-177 and serine-538 were required for the PGC-1α-dependent induction of the PGC-1α promoter (Jager, et al., 2007).

AMPK may also regulate PGC-1α expression by phosphorylating the transcriptional repressor histone deacetylase 5 (HDAC5). During its inactive form, HDAC5 interacts with MEF2 and modifies the chromosome structure to one that silences transcription (Baar and McGee, 2008; McGee, 2007; McGee and Hargreaves, 2008; McKinsey, et al., 2000; McKinsey, et al., 2001). Phosphorylation of HADC5 at site Ser259 and Ser498 disrupts the interaction between MEF2 and HDAC5 and leads to the nuclear export of HDAC5 by the 14-3-3 chaperone protein. This results in subsequent chromatin remodeling and MEF2 transcriptional activity is enhanced to increase PGC-1α expression (Baar and McGee, 2008; Czubryt, et al., 2003; McGee, 2007; McGee and Hargreaves, 2008; McKinsey, et al., 2000; McKinsey, et al., 2001) (Figure 1.5). AMPK has been shown to be able to phosphorylate HDAC5 at Ser259 and Ser498 (McGee, et al., 2008a) and prolonged submaximal exercise has also been shown to increase AMPKα2 content in the nucleus of human muscle cell (McGee, et al., 2003). As such the AMPK-HDAC5-MEF2 axis
has been proposed as one of the pathways by which AMPK regulate PGC-1α and subsequent mitochondrial biogenesis.

As noted above, the CRE binding proteins are needed for the transcription of PGC-1α. A recent study by Thomson et al. (2008) demonstrated that the CRE binding protein (CREB) is a direct downstream targets of AMPK. This result indicates that AMPK may also increase PGC-1α transcription by phosphorylating CREB to induce mitochondrial biogenesis in response to exercise (Figure 1.6).

On the other hand, p38 MAPK is believed to modulate PGC-1α activity by two putative mechanisms. PGC-1α activity can be regulated by p160myb, a repressor protein that can powerfully inhibit PGC-1α function by blocking it’s interactions with transcription factors (Fan, et al., 2004). The phosphorylation of PGC-1α by p38 MAPK has been shown to disrupt this association between p160myb and PGC-1α (Fan, et al., 2004). Consequently, PGC-1α can be recruited to transcription factors to increase it’s transcriptional activity (Fan, et al., 2004) (Figure 1.6). In a recent study, Akimoto et al. (2005) demonstrated that the p38 MAPK mediate increased in PGC-1α promoter activity was enhanced by increased expression of the downstream transcription factor activating transcription factor 2 (ATF2), and completely blocked by a dominant negative ATF2. These results indicated that ATF2 is required for the increased in the transcription of PGC-1α through p38 MAPK activation and further provide another mechanistic explanation by which exercise and thus p38 MAPK activation may mediate mitochondrial biogenesis (Akimoto, et al., 2005) (Figure 1.6).
In summary, the introduction of molecular biology techniques into exercise metabolism study has made possible the identification of cellular and molecular factors that may provide the link between endurance training and mitochondrial biogenesis and how these factors are regulated by nutrient availability and/or the interactive effects of nutrient availability and endurance training.
Figure 1.5  Schematic diagram showing the proposed mechanism by which AMPK increase PGC-1α transcription through phosphorylation of histone deacetylase 5 (HDAC5). αβγ, 5’ AMP activated protein kinase; MEF2, myocyte enhancer factor 2; PGC-1α, Peroxisome proliferator-activated receptor-γ coactivator-1α; 14-3-3, 14-3-3 chaperone protein [adapted from Baar and McGee (2008)].
Figure 1.6  Putative downstream signalling pathways of AMPK and p38 MAPK in mediating skeletal muscle mitochondrial biogenesis. Bars denote inhibition and arrows denote activation. CHO, carbohydrate; αβγ, AMP-activated protein kinase; HDAC5, histone deacetylase 5; CREB, cyclic AMP response element binding protein; ATF2, activating transcription factor 2; p160myb, p160 myb binding protein; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator-1α.
Chapter Two

Aims of Thesis
Aims of thesis

The overall aim of the experiments undertaken for this thesis was to investigate how altering skeletal muscle fuel stores interacts with endurance exercise to modify skeletal muscle adaptation. A specific aim was to study some of the potential cell signalling events that underlie the observed perturbations in muscle metabolism. In this regard, endogenous CHO and lipid stores in muscle were altered by manipulating dietary intake and/or the training stimulus. The scope of the investigations undertaken for this thesis was limited to endurance exercise training adaptation in well-trained humans.

The literature review (Chapter One) provides an overview of the role of nutrient availability and exercise training on skeletal muscle adaptation. Subsequently, several nutritional strategies that have been used to modify fuel stores in muscle with a view to enhancing exercise capacity and/or training adaptation are discussed. In addition, potential mechanisms that may influence skeletal muscle responses to the various nutrient-training interventions are also discussed.

In the first experiment described in Chapter Three, a short term model of ‘dietary periodisation’ known as fat adaptation with carbohydrate restoration was utilised to study skeletal muscle adaptation. Well-trained cyclists undertook their normal high volume training while consuming either a high carbohydrate or high fat diet for 5 days followed by 1 day of ‘carbohydrate restoration’ and rest. The main objective of this experiment was to investigate potential mechanisms that underlie the previously observed increases in rates of whole-body fat oxidation that persist after ‘fat adaptation and CHO restoration’. The hypothesis to be tested in this study was that a short-term period of ‘fat adaptation’ followed by carbohydrate restoration would
increase resting muscle triglyceride concentrations and that changes in lipid availability \textit{per se} would modify AMPK signalling to increase the rates of fat oxidation at rest and during submaximal exercise.

In the second study described in Chapter Four, well-trained endurance cyclists/triathletes undertook a chronic (3-wk) training programme in which subjects either trained once everyday for 6 consecutive days or twice every second day. The main objective of this study was to investigate the effects of these two different training regimens on 1) skeletal muscle fuel storage profile, 2) training capacity and endurance performance, 3) whole-body substrate metabolism during submaximal exercise and 4) several mitochondrial enzymes and signalling proteins with putative roles in promoting training adaptation. The hypothesis to be tested in this study was that well-trained athletes would have maximised their training adaptation and that further gains in skeletal muscle adaptation and performance would be minimal, irrespective of whether they trained with low or normal levels of muscle glycogen.

In the final experiment described in Chapter Five, well-trained endurance cyclists/triathletes participated in an acute exercise study in which they undertook either a single session of training once daily or trained twice the same day. The primary aim of this experiment was to elucidate the potential mechanisms that may help explain the superior training adaptation observed in the previous study (Chapter Four) in which training twice every second day promoted selected muscle adaptations to a greater extent than training daily. The hypothesis to be tested in this study was that performing intense endurance exercise in the face of low muscle glycogen concentration
would result in increased activation of AMPK and p38 MAPK and some of their downstream substrates.
Chapter Three

Fat adaptation followed by carbohydrate restoration increases AMPK activity in skeletal muscle from trained humans

3.1 Introduction

Short-term (<1 wk) manipulation of dietary macronutrient intake is associated with marked changes in skeletal muscle gene expression (Arkinstall, et al., 2004; Cameron-Smith, et al., 2003; Jump and Clarke, 1999), substrate stores (Zderic, et al., 2004), metabolic flux and fuel oxidation (Coyle, et al., 2001; Horowitz, et al., 1997; 1999). Exercise training also results in striking modifications in muscle gene expression (Freyssenet, 2007), energy reserves, and the relative contribution of fuels to the energetic demands of muscle (Coggan, et al., 1990). Accordingly, the extent to which acutely altering substrate availability might modify the training impulse has been a key research area among exercise physiologists and sport nutritionists for several decades [for review see Hawley et al. (2006)]. Indeed, evidence is accumulating that nutrient manipulation can serve as a potent modulator of many of the acute responses to both endurance (Hansen, et al., 2005) and resistance exercise (Churchley, et al., 2007; Creer, et al., 2005).

During recent years, a series of independent but related studies have been performed by several investigators to examine the effects of a practical “dietary periodisation” strategy in well-trained endurance athletes on selected aspects of metabolism and exercise performance (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001; Stellingwerff, et al., 2006; Stepto, et al., 2002). The results of these studies have shown that short-term (5 d) exposure to a high-fat diet while undertaking high-volume, intense training followed by 1 d of rest and “carbohydrate restoration” results in greater rates of whole-body fat oxidation and decreased rates of muscle glycogenolysis during submaximal [70% peak O\textsubscript{2} uptake (VO\textsubscript{2peak})] cycling than when subjects undertake the same training and consume an isoenergetic high carbohydrate diet (Burke, et al., 2000; Stellingwerff, et al., 2006). The decreased rate of muscle glycogenolysis during exercise
following fat adaptation with carbohydrate restoration can, in part, be explained by decreased pyruvate oxidation via PDH flux (Stellingwerff, et al., 2006). However, it is not clear what precise metabolic signal(s) accounted for the marked shifts in substrate utilisation during exercise. In this regard, it was suggested that AMP-activated protein kinase (AMPK) may provide a direct link between intracellular signalling events and subsequent substrate selection in exercising muscle (Hardie and Sakamoto, 2006; Winder, 2001). In support of this hypothesis, diet-exercise manipulation that result in low muscle glycogen content are associated with increased resting AMPK activity (Steinberg, et al., 2006; Wojtaszewski, et al., 2003) and elevated phosphorylation of AMPK’s downstream target, β-acetyl-CoA carboxylase (ACCβ) Ser221 compared to high glycogen stores (Wojtaszewski, et al., 2003). Furthermore, the degree of AMPK activation during submaximal exercise was also shown to be dependent on the fuel status of the contracting musculature, with AMPK activity elevated to a greater extent in muscle with low compared to high glycogen levels (Wojtaszewski, et al., 2003).

While the findings of Wojtaszewski et al. (2003) imply that glycogen availability per se may modulate AMPK activity, it cannot be ruled out that the diet-exercise regimens used to differentiate glycogen concentration in those studies also resulted in significant changes in muscle lipid availability [i.e. increased muscle triacylglycerol (TG)]. Accordingly, the observed changes in AMPK activation may have been more a consequence of lipid-induced rather than carbohydrate-induced effects on this enzyme. In this regard, Lessard et al. (2007) recently showed that endurance training in high-fat fed rats was associated with increased AMPKα1 activity, whereas AMPKα2 activity was elevated by high-fat feeding alone and was not increased further by exercise training (Lessard, et al., 2007). Accordingly, the aim of the current study was
to investigate the effect of a diet-exercise protocol known to be associated with elevated rates of fat oxidation independent of muscle glycogen availability on AMPK signalling at rest and during subsequent submaximal exercise. It was hypothesised that fat adaptation followed by carbohydrate restoration would increase resting muscle triglyceride concentrations and that changes in lipid availability per se would modify AMPK signalling at rest and during exercise.

3.2 Methods

3.2.1 Subjects and preliminary testing

Eight endurance-trained male cyclists or tri-athletes [body mass (BM) 70.2 ± 1.8 kg; age 31.6 ± 2.3 yr; peak O$_2$ uptake (VO$_{2peak}$) 61.5 ± 1.5 mL/kg/min, peak sustained power output (PPO) 339.9 ± 5.8 W; values are mean ± SE] were recruited to participate in this study which was approved by the Human Research Ethics Committee of RMIT University. Subjects were fully informed about the possible risks of all procedures before providing their written consent. One week prior to experimental testing, each subject undertook an incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode, Groningen, The Netherlands). The initial testing protocol has been described in detail previously (Hawley and Noakes, 1992). In brief, the subjects started the test at an intensity equivalent to 3.33 W/kg BM. This initial intensity was maintained for 150 s and thereafter was increased by 50 W for a further 150 s. After the second stage the exercise intensity was increased by 25 W every 150 s until the subjects reached fatigue, which always coincided with either a drop in rate of greater than 10 rpm, a respiratory exchange ratio (RER) greater than 1.10, or both (Hawley and Noakes, 1992). During the maximal test and the subsequently described experimental trials, subjects breathed through a Hans Rudolph two way non-rebreathing valve and mouthpiece attached to a gas analysis system (Parvomedics, Utah,
U.S.A) interfaced to a computer, which calculated the instantaneous rates of O₂ consumption (\(\text{VO}_2\)), CO₂ production (\(\text{VCO}_2\)), minute ventilation (\(\text{VE}_{\text{STPD}}\)), and the RER every 15 s from conventional equation (Péronnet and Massicotte, 1991). Before each maximal test and all experimental trials, the analyzers were calibrated with commercially available gases of known O₂ and CO₂ content. \(\text{VO}_{2\text{peak}}\) was defined as the highest O₂ uptake a subject attained during any 60 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final noncompleted work rate multiplied by 25 W. This value was used to determine the power output corresponding to 70% of each subject’s \(\text{VO}_{2\text{peak}}\) (63% of PPO) to be used in the subsequently described experimental trials. The maximal test session and all experimental trials were performed under standard laboratory condition (18-22°C, 40-50% relative humidity) and subjects were fan cooled during all these sessions.

### 3.2.2 Overview of study design

The experimental protocol is shown in Figure 3.1 and has been described in detail previously (Burke, et al., 2000). In brief, each subject undertook two 7-day trials in a randomised, crossover design separated by a 2-wk washout period. The interventions consisted of prescribed supervised training while undertaking either a 5-day high-fat diet followed by a 1-day of high CHO restoration (FAT-adapt) or a 6-day high-CHO diet (HCHO). During FAT-adapt, the subjects were prescribed a high-fat (4.6 g/kg/d fat, 68% of energy), low CHO (2.5 g/kg/d CHO, 17% of energy) diet supplying 0.25 MJ/kg BM. HCHO was an isoenergetic diet providing 10.3 g/kg/d and 70% of energy from CHO and 1.0 g/kg/d and 18% of energy from fat. Protein content was maintained at 2.3 g/kg/d during both trials and diets were constructed to maximize, or at least match, absorbable energy. Fiber intake was kept to a daily mean intake of <50 g and matched to
within 5 to 10 g each day between dietary treatments. All meals and snacks were supplied to
subjects, with diets being individualized for food preferences as well as BM. Subjects received
their food in pre-prepared packages and were required to keep a food checklist to note their
compliance to the dietary instructions and their intake of any additional food or drinks. Every two
days they met with a dietician to receive new food parcels and check their adherence to the
previous days’ diet. The high-CHO diet prescribed in this study is similar in composition to that
recommended by sports nutritionists for endurance trained athletes (Burke, 2003). However, the
high-fat diet is far removed from the nutritional energy sources typically consumed by our
subjects. While an ad libitum diet group may provide insight into diet-training interactions, the
addition of such a group was not considered within the current experimental design.

On the morning of day 1 and day 5 of each trial, subjects reported to the laboratory after a 12- to
14-h overnight fast and undertook a 20-min steady state ride at 70% of VO$_{2peak}$. Pulmonary gas
exchange data were collected for the last 5-6 min to calculate the rates of whole body fat and
CHO oxidation. This ride was immediately followed by a high intensity interval training session
consisting of 8 repetitions of 5 min work bouts at 85% of VO$_{2peak}$ with 1 min of recovery at 100
W. The metabolic demands associated with this interval training session have been described in
detail previously (Stepto, et al., 2001). The intent of this ride was to lower the muscle glycogen
concentrations on the first day and initiate a rapid differentiation between dietary treatments on
the basis of their ability to restore depleted muscle glycogen stores. On day 6 of both the FAT-
adapt and HCHO trials, subjects were required to rest and ingest a high carbohydrate diet (10.3
g/kg BM).
Figure 3.1. Overview of the study design and experimental trial. CHO, carbohydrate; VO$_{2peak}$, peak oxygen uptake.
3.2.3 Experimental trial

On the morning of day 7, subjects returned to the laboratory after a 12- to 14-h overnight fast to undertake a 1 h steady state ride at 70% of VO$_{2peak}$. On arrival in the laboratory, a single leg was prepared for muscle biopsies, and two incisions were made 2–3 cm apart along the vastus lateralis muscle. A resting muscle sample was then taken using the percutaneous biopsy technique with suction applied. After resting for 10 min, subjects began the 60-min steady state ride and a second biopsy was obtained within 10 seconds on completion of the ride while subjects remained seated on the ergometer (Figure 3.1). All muscle biopsies were rapidly frozen in liquid N$_2$ within seconds of the designated time point for collection and stored at -80ºC until subsequent analysis.

3.2.4 Analytical Procedures

Rates of fat and CHO oxidation

Whole body rates of carbohydrate and fat oxidation (g/min) were calculated from the respiratory data collected during the last 5-6 min of the 20-min steady state ride on day 1 and day 5 and during the last 5 minutes of every 20 minutes of the steady state ride at 70% VO$_{2peak}$. The calculations were made from VCO$_2$ and VO$_2$ measurements, assuming a nonprotein RER value, according to the following equations (Péronnet and Massicotte, 1991).

CHO oxidation = 4.585 VCO$_2$ - 3.226 VO$_2$

Fat oxidation = 1.695 VO$_2$ - 1.701 VCO$_2$

Total fat and carbohydrate oxidation during the 60 min steady state ride were estimated by calculating the area under the oxidation (g/min) versus time curves for each subject. Rates of
CHO oxidation (μmol/kg/min) were determined by converting the rate of CHO oxidation (g/min) to its molar equivalent assuming 6 mol of O\textsubscript{2} are consumed and 6 mol of CO\textsubscript{2} produced for each mole (180 g) oxidised. Rates of fatty acid oxidation (μmol/kg/min) were determined by converting the rate of triglycerol oxidation (g/min) to its molar equivalent assuming the average molecular weight of human triglyceride to be 855.3 g/mol and multiplying the molar rate of triglyceride oxidation by 3, because each molecule contains three molecules of fatty acids.

**Muscle glycogen and triacylglycerol concentration**

Approximately 40-50 mg of muscle was freeze-dried and powdered with all visible blood and connective tissue removed. The freeze-dried muscle sample was then divided into two aliquots. The first aliquot (~3 mg) was extracted with 250 μL of 2 M hydrochloric acid, incubated at 100°C for 2 hr and then neutralized with 750 μL of 0.67 M sodium hydroxide. Glycogen concentration was determined via enzymatic analyses with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) and was expressed as micromoles of glycogen per gram of dry weight μmol/g dry wt. (Churchley, et al., 2007).

The second aliquot (~7 mg) was used for the determination of muscle triacylglycerol content, as previously described (Frayn and Maycock, 1980). In brief, lipid was extracted by Folch extraction (Folch, et al., 1957), the triacylglycerol was then saponified in an ethanol/potassium hydroxide solution at 60°C, and glycerol content was determined fluorometrically and expressed as μmol/g dry wt.
**AMPK Signalling**

Approximately 60 mg of wet muscle were homogenised in buffer A (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and the homogenate was then centrifuged at 20,000 g for 25 min. The supernatant was aliquoted and the total protein concentration was determined by bicinchoninic acid method (Pierce Chemical, Rockford, IL). The aliquots were then stored at -80°C until further analysis.

**Immunoprecipitation**

Approximately 2.5 mg of protein from the supernatants were incubated with AMPKα1 or AMPKα2 antibody bound protein A-Sepharose beads (6 MB; Amersham Biosciences, Uppsala, Sweden) for 2 h at 4°C. The polyclonal antipeptide antibodies to AMPKα1 and AMPKα2 were raised to non-conserved regions of the AMPKα1 (rat 231–251) and AMPKα2 isoforms (rat 351–366). The immunocomplexes were washed with PBS and suspended in 50 mM Tris.HCl (pH 7.5) buffer for the AMPK activity assay. The AMPK activities in the immune complexes were measured in the presence of 200µM AMP. Activities were calculated as picomoles of phosphate transferred to the SAMS peptide per minute per milligram of protein subjected to immunoprecipitation (pmol/min/mg). The post-AMPK immunoprecipitation supernatants were then incubated in streptavidin-Sepharose high-performance beads (Amersham Biosciences) for 1 h at 4°C for affinity purification of ACC.
**Western blotting**

The affinity-purified ACC fraction was electrophoresed on 7.5% SDS-PAGE and detected by immunoblotting with anti-phospho-Ser^{221}-ACC polyclonal antibody. The blots were then stripped (50 mM Tris.HCl, 2% SDS, 115 mM β-mercaptoethanol) at 55°C for 20 min, blocked, and incubated with horseradish peroxidase conjugated streptavidin (Amersham Pharmacia Biotech UK, Little Chalfont, UK) to determine the total ACC content. Aliquots of the immunoprecipitated AMPKα1 and AMPKα2 proteins were electrophoresed on 10% SDS-PAGE and detected by immunoblotting with antibodies specific for the AMPKα1 and AMPKα2 isoforms. The post-AMPK immunoprecipitation supernatants containing 60 μg of total protein were electrophoresed on SDS-PAGE [Acrylamide concentration either 15% (GLUT 4) or 10% (FAT CD/36, PGC1α) respectively] and detected by immunoblotting with antibodies specific for GLUT4 (Biogenesis, UK 4670-1704), FAT/CD36 (AbCam, Cambridge, UK) or PGC-1 (Millipore, Inc. AB3242). The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry. α-tubulin was used as a loading control to ensure that the protein content was the same in all wells.

3.2.5 **Statistical Analysis**

Data from the two experimental trials were compared by using a two-factor (diet and time) analysis of variance (ANOVA) with repeated measures. Separate analyses were undertaken to compare data from 20-min rides on day 1, 5, and 7 and data collected at different time points during the experimental ride. Holm-Sidak post hoc tests were undertaken when ANOVA revealed a significant interaction. Muscle glycogen and muscle TG concentration between trials were compared by using Student’s t-tests. Relationship between muscle fuel stores (muscle glycogen
and muscle TG) and AMPK activities were studied using Pearson product-moment correlation coefficient. All values are expressed as means and standard error (SE) with the critical level of significance established at $P < 0.05$.

### 3.3 Results

#### 3.3.1 Substrate oxidation during exercise

Figure 3.2A shows the rates of whole body CHO oxidation during 20-min of cycling at 70% VO$_{2\text{peak}}$ (20-min SS) on day 1, day 5 and day 7. Rates of CHO oxidation during the 20-min SS were similar during both trials on Day 1. There was a significant effect of diet on rates of CHO oxidation such that HCHO was higher than FAT-adapt at all time points after baseline ($P < 0.05$). During HCHO trial, 1 day of rest on Day 6 increased the rates of whole body CHO oxidation during 20-min SS significantly compared to baseline ($P < 0.05$). During FAT-adapt, 5 days of high fat diet and exercise training decreased the rates of whole body CHO oxidation such that it was significantly lower than all the other time points ($P < 0.05$).

Figure 3.2B shows the rates of whole body fat oxidation during 20-min of cycling at 70% VO$_{2\text{peak}}$ (20-min SS) on day 1, day 5 and day 7, along with the average RER data during the 1 h steady-state ride (60-min SS) on day 7 (Figure 3.2C). Rates of fat oxidation during the 20-min SS were similar during both trials ($P < 0.05$) at baseline (Day 1). The effect of diet on rates of fat oxidation was significant at all time points after baseline, with FAT-adapt being higher than HCHO ($P < 0.05$). One day of high CHO diet and rest decreased rates of fat oxidation, such that on day 7 values were not different from day 1 (30.20 ± 3.42 vs. 25.79 ± 3.72 µmol/kg/min respectively; $P < 0.05$). During HCHO, rates of fat oxidation were similar on day 1 and day 5.
(23.03 ± 3.61 vs. 18.85 ± 2.61 µmol/kg/min; \(P < 0.05\)) but decreased on day 7 compared to day 1
(23.03 ± 3.61 vs. 14.44 ± 1.19 µmol/kg/min; \(P < 0.05\)). The average RER over the 1 h steady
state ride on day 7 was also lower during FAT-adapt (0.87 ± 0.004 vs. 0.93 ± 0.004; \(P < 0.01\);
Figure 3.2C) such that fat oxidation accounted for 40.3 ± 3.6 % of total fuel oxidation in this trial
compared to 21.9 ± 1.5 % in HCHO (\(P < 0.05\)).

### 3.3.2 Glycogen and Muscle TG

Table 3.1 displays muscle glycogen concentrations before and after the two experimental trials.
As intended, there were no differences in pre-exercise muscle glycogen levels between dietary
treatments (547 ± 40 and 694 ± 55 µmol/g dry wt. for FAT-adapt and HCHO respectively).
Glycogen utilisation during 60-min SS was significantly lower after FAT-adapt (179 ± 35 vs. 264
± 45 µmol/g dry wt., \(P < 0.05\)).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Day-7 Pre-exercise</th>
<th>Day-7 Post-exercise</th>
<th>Utilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAT-adapt</td>
<td>547 ± 40</td>
<td>369 ± 22 *</td>
<td>179 ± 35</td>
</tr>
<tr>
<td>HCHO</td>
<td>694 ± 55</td>
<td>460 ± 54 *</td>
<td>264 ± 45 §</td>
</tr>
</tbody>
</table>

Values are means ±SE given in µmol/g dry wt.; \(n \geq 7\)/group. * Significantly different from
pre-exercise, \(P < 0.05\). § Significantly greater than FAT-adapt.
Figure 3.2  (A) Rates of whole body CHO oxidation estimated during 20-min of cycling at 70% VO_{peak} on day 1, day 5 and during 1 h of steady state cycling at 70% VO_{peak} on day 7 (B) Rates of whole-body fat oxidation estimated during 20-min of cycling at 70% VO_{peak} on day 1, day 5 and during 1 h of steady state cycling at 70% VO_{peak} on day 7. #, Significantly different from HCHO trial (P < 0.05). †, Significantly different from day 1 (P < 0.05). *, Significantly different from all time points within FAT-adapt (P < 0.05). (C) Average RER values during the 1 h steady-state ride at 70% VO_{peak} on day 7 (P = 0.002). Values are mean ± SE for 8 subjects.
3.3.3 Muscle TG

Figure 3.3 displays muscle TG concentrations before and after 60-min steady state cycling at 70% VO$_{2\text{max}}$. Muscle TG concentrations were higher on day 7 after FAT-adapt compared to the HCHO trial (69 ± 10.2 vs. 39 ± 3.4 µmol/g dry wt.; $P < 0.01$). Post-exercise muscle TG levels were similar between FAT-adapt and HCHO trial (Figure 3.3). There was a small but non-significant increase in muscle TG concentration with HCHO after exercise.

![Figure 3.3](image)

**Figure 3.3** Muscle TG concentrations before and after exercise (60-min steady-state ride at 70% VO$_{2\text{peak}}$) on day 7 during high carbohydrate (HCHO) and fat adaptation with carbohydrate restoration (FAT-adapt) trials. #, Significantly different from HCHO ($P < 0.01$). Values are mean ± SE; n ≥ 7/group.

3.3.4 AMPK α1 and α2 activity

Figure 3.4 displays resting (pre-exercise) and post-exercise AMPK signalling (AMPKα1 and α2 activity and the phosphorylation of ACC at Ser$^{221}$) after the two dietary treatments and resting
total protein content of AMPKα1, AMPKα2 and ACC. Resting AMPK α1 and α2 activities (Figure 3.4 A and B) were higher after FAT-adapt compared to HCHO (p=0.02 and p=0.05 respectively). There were no differences in the protein content of either AMPKα1 or -α2 in response to either treatment (Figure 3.4C). Exercise increased AMPKα1 activity (Figure 3.4A) after HCHO (p=0.02) but did not have any further effects on either AMPKα1 or α2 activity after FAT-adapt. AMPK α1 and α2 activity post-exercise were not different between dietary treatments.

There was a significant relationship \( (r = 0.53; \ P < 0.05) \) between resting muscle TG concentration and AMPKα2 activity (Figure 3.5A). When this relationship was expressed as the difference in resting muscle TG for each subject between the two dietary treatments versus the difference in AMPKα2 activity between treatment, the correlation improved to \( r = 0.82 \ (P < 0.05; \ Figure \ 3.5B) \). Resting muscle glycogen concentration was significantly associated with AMPKα1 \( (r = -0.51; \ P < 0.05; \ Figure \ 3.5C) \) but not AMPKα2 activity. There was a trend for a relationship between muscle TG utilisation and changes in AMPKα2 activity \( (r = 0.52; \ P = 0.06; \ Figure \ 3.5D) \) but no relationship between muscle TG utilisation and AMPKα1 activity.

### 3.3.5 Phosphorylation of ACC at Ser\(^{221}\) (pACC Ser\(^{221}\))

The phosphorylation of the AMPK downstream target ACC at Ser\(^{221}\) (relative to total protein content) on day 7 tended to be higher at rest after FAT-adapt compared to HCHO \( (P = 0.09) \). Exercise increased the pACC Ser\(^{221}\) during both HCHO \( (P = 0.042) \) and FAT-adapt \( (P = 0.008) \) with the post-exercise pACC Ser\(^{221}\) being higher during the FAT-adapt compared to the HCHO trial \( (P = 0.02; \ Figure \ 3.4D) \).
Figure 3.4 AMPK signalling before and after exercise on day 7 during high carbohydrate (HCHO) and fat adaptation with carbohydrate restoration (FAT-adapt) trials. (A) AMPK α1 activity, (B) AMPK α2 activity, (C) Relative levels of resting (pre-exercise) total protein content of AMPK α1, AMPK α2 and ACC as quantified by western blot analysis and densitometry, (D) Phosphorylation of ACC at Serine 221 relative to total ACC protein content (pACC Ser\textsuperscript{221}). Significant differences between groups \((P < 0.05)\) are indicated by the \(P\) values listed on the figure. Values are mean ± SE for 8 subjects.
AMPKα2 activity between muscle triglyceride concentration and differences in resting AMPKα2 activity (n = 14); (B) Differences in resting muscle triglyceride concentration and differences in resting AMPKα2 activity between trials (n = 6 pairs); (C) resting muscle glycogen concentration and resting AMPKα1 activity (n = 15) between trials; (D) Changes in AMPKα2 activity during exercise and muscle TG utilisation during exercise (n = 7 pairs).

Figure 3.5 Scatter plots showing the relationship between (A); resting muscle triglyceride concentration and resting AMPKα2 activity (n = 14); (B) Differences in resting muscle triglyceride concentration and differences in resting AMPKα2 activity between trials (n = 6 pairs); (C) resting muscle glycogen concentration and resting AMPKα1 activity (n = 15) between trials; (D) Changes in AMPKα2 activity during exercise and muscle TG utilisation during exercise (n = 7 pairs).
3.3.6 Protein expression

Total protein abundance for FAT/CD36, GLUT 4 and PGC1 were not different between trials (Figure 3.6).

![Representative western blots showing total protein concentration for FAT/CD36, GLUT4 and PGC1 before (pre) and after (post) exercise on day 7 during HCHO and FAT-adapt trials.](image)

Figure 3.6 Representative western blots showing total protein concentration for FAT/CD36, GLUT4 and PGC1 before (pre) and after (post) exercise on day 7 during HCHO and FAT-adapt trials.
3.4 Discussion

This study used a human model of short-term (5 days) adaptation to a high-fat diet followed by 1 day of carbohydrate restoration (i.e., a high carbohydrate diet) in well-trained athletes undertaking high-volume, intense training to study the effects of diet-exercise interactions on muscle signalling and metabolism. The novel findings from the present study were that despite the brevity of the intervention period, 1) the FAT-adapt protocol resulted in elevated resting muscle triglyceride concentrations that were independent of glycogen availability, 2) changes in muscle triglyceride levels were associated with increased basal AMPK activity without concomitant changes in protein levels, and 3) FAT-adapt attenuated the exercise-induced rise in AMPKα1 and AMPKα2 activity compared to an isoenergetic high carbohydrate diet.

In accordance with previous investigations (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001; Stellingwerff, et al., 2006), this study again report that rates of fat oxidation during 60 min of submaximal cycling (70% of VO$_{2\text{peak}}$) were elevated ~2-fold after fat adaptation followed by carbohydrate restoration compared to when subjects performed identical training and consumed an isoenergetic high carbohydrate diet throughout the 6 day intervention period (Figure 3.2). Furthermore, muscle glycogen utilisation was significantly lower after FAT-adapt (Table 3.1). In several earlier studies performed utilising similar dietary perodization model (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001; Stellingwerff, et al., 2006), it was not clear what metabolic signal(s) accounted for the shift in muscle substrate use during exercise. The results of the present study show that one of the residual effects of the short-term high-fat diet was to increase resting muscle TG stores (Figure 3.3). The resting TG levels observed in these well-trained athletes after just 5 d of consuming a high-fat diet are in close agreement with those
reported by Helge et al. (2002) for previously untrained subjects who completed 7 wk of endurance training while consuming a high-fat diet (Helge, et al., 2002). They are also similar to the values of Zderic et al. (2004) for endurance-trained cyclists after 2 d of a high-fat diet and Zehnder et al. (2006) in muscle from trained athletes after just 1.5 days of lipid supplementation (Zehnder, et al., 2006). Taken collectively, these findings suggest that muscle TG stores are prone to rapid fluctuations in response to changes in nutrient composition and physical activity levels.

In one of the previous studies (Cameron-Smith, et al., 2003), it was shown that 5 days of a high-fat diet coupled with a rigorous training programme resulted in a small (17%) but significant increase in the protein abundance of fatty acid translocase (FAT/CD36). These data (Cameron-Smith, et al., 2003) indicated that an increase in dietary lipid availability was, in part, responsible for the rapid up-regulation of FAT/CD36 protein expression, suggesting a role for this FA transporter in facilitating greater skeletal muscle FA uptake. In that investigation, the effects of 5 d of a high-fat diet on FAT/CD36 was examined without a 1-day carbohydrate restoration phase. In the present study, FAT/CD36 protein content was measured after fat adaptation followed by 1 day of rest and a high carbohydrate diet. Under these dietary conditions, there were no differences in the abundance of this FA transporter detected between the fat adaptation protocol and the high carbohydrate diet intervention. To the best of our knowledge, no previous studies have measured GLUT4 or PGC1 protein expression in response to the short-term fat adaptation and carbohydrate restoration protocol utilised in the present study. However, Steinberg et al. (2006) reported higher skeletal muscle GLUT4 mRNA expression immediately after an acute bout of endurance exercise commenced with low (versus normal) glycogen content. With regard to PGC1, Sparks et al. (Sparks, et al., 2005) reported that PGC1α mRNA was reduced by 20% in muscle from healthy
young males following 3 days of a high fat diet, whereas Mortensen et al. (2007) found no differences in PGC-1α mRNA expression after a chronic training intervention in previously untrained subjects in which 50% of the training sessions were performed under conditions of low starting muscle glycogen concentration.

A second important finding from the present study was that the high-fat diet was accompanied by increased AMPKα1 and α2 activity (Figure 3.4, Panels A and B). Wojtaszewski et al. (2003) have also demonstrated that resting AMPK activity is sensitive to fuel status (Wojtaszewski, et al., 2003). These workers reported that AMPK activities for both α1 and α2 isoforms were higher in the face of low- (~160 mmol/kg dry wt.) compared to high- (~900 mmol/kg dry wt.) resting muscle glycogen content. While their results (Wojtaszewski, et al., 2003) suggest a relationship between muscle glycogen content and AMPK activity, it is unlikely that altered muscle glycogen availability is the only factor regulating muscle AMPK activity. The diet-exercise intervention used to differentiate resting muscle glycogen stores in the study of Wojtaszewski et al. (2003) is also likely to have resulted in elevated muscle triacylglycerol levels, particularly as arterial plasma FFA concentrations were significantly higher in the low- versus high-glycogen condition. Although plasma FA levels were not measured in the current investigation, it’s concentrations at rest has previously been shown to be similar between the two diet interventions (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001).

A recent study reported that AMPKα2 but not -α1 activity was increased in rodent skeletal muscle in response to chronic high-fat feeding (Lessard, et al., 2007). Of note in that study was the observation that AMPKα1 activity was only increased in high fat-fed rodents when they
concurrently undertook an intense endurance training programme with a high-fat diet (Lessard, et al., 2007). At the time Lessard et al. (2007) suggested that there may be distinct roles for the AMPK -α subunit isoforms in skeletal muscle, with AMPKα1 activity being linked to exercise training-induced adaptations and muscle glycogen storage and AMPKα2 activity being responsive to increased lipid availability (Lessard, et al., 2007). The present study provides further data in support of this premise. A significant relationship between resting AMPKα1 activity (but not -α2) and muscle glycogen content ($r = -0.51$, $P < 0.05$) was observed. In contrast, AMPKα2 (but not α1 activity) was positively associated with resting muscle triglyceride content ($r = 0.53$, $P < 0.05$). Perhaps the most robust evidence to corroborate the isoform-specific modification of resting AMPK activity and muscle lipid status comes from the paired data from each subject’s resting biopsy samples: compared to a high carbohydrate diet, fat adaptation significantly increased muscle triglyceride concentration (Figure 3.3), with such changes being strongly correlated to difference in basal AMPKα2 activity ($r = 0.82$, $p<0.05$). Raney and Turcotte (2006) have also reported a positive association between fatty acid uptake and oxidation and AMPKα2 activity in the perfused rat hindlimb model. However, it should be noted that correlational data cannot determine causality and it is possible that factors other than muscle lipid status may play a role in modifying AMPKα2 activity. In this regard, while resting muscle glycogen concentrations were not statistically significant between the two dietary groups, values in the high-carbohydrate trial were, on average, 27% higher than after the high-fat diet. It is well accepted that pre-exercise muscle glycogen availability influences subsequent rates of muscle glycogenolysis (Hargreaves, et al., 1995; Hargreaves and Richter, 1988). Indeed, it is likely that a 25-30% difference in resting glycogen content is of physiological significance (Hargreaves, et al., 1995).
While it is tempting to attribute a causal role for altered muscle substrate availability (i.e. glycogen and/or triglyceride stores) on subsequent changes in AMPK signalling, it should be acknowledged that differences in AMPK responses in the present study could, in part, be due to the well-trained subjects performing prolonged, strenuous training on a high-fat diet (i.e., the interactive effects of diet and exercise). In this regard, Hansen et al. (2005) have previously reported that compared to daily training with normal glycogen reserves, commencing 50% training sessions with low muscle glycogen (and presumably higher triglyceride) levels resulted in a more pronounced increase in citrate synthase activity. The potential mechanism(s) for this augmented training response after ‘low glycogen’ training is hard to define, but it is possible that commencing exercise with reduced glycogen (i.e. high-fat) availability may promote training adaptations through perturbation in circulating systemic factors (i.e. increased catecholamines), altered muscle substrate availability or a combination of both. Although catecholamine levels were not measured during training in the present study, Hansen et al. (2005) have reported that the catecholamine response to exercise performed with low muscle glycogen levels are higher than when exercise undertaken with normal glycogen stores, demonstrating a higher stress response. Further work will be needed to determine the precise roles of local (muscle) versus systemic factors in modifying AMPK responses to diet/exercise manipulations.

As noted previously, one of the residual effects of 5 d of a high-fat diet in combination with intense training is that rates of whole-body fat oxidation during submaximal exercise remain elevated above those observed after a high carbohydrate diet, even when carbohydrate stores are restored (Burke, et al., 2000; Carey, et al., 2001; Stellingwerff, et al., 2006). Accordingly, one would predict that the pattern of activation of the downstream target of AMPK, ACCβ would
reflect such perturbations. This is indeed the case. \( \text{pACC}\beta \text{ Ser}^{221} \) tended to be higher at rest after fat adaptation compared to the high carbohydrate diet \((P = 0.09, \text{Figure 3.4 D})\), and while there was an exercise-induced increase in \( \text{pACC}\text{ Ser}^{221} \) for both treatments, the phosphorylation state of \( \text{ACC}\beta \text{ Ser}^{221} \) was significantly higher at the end of exercise after fat adaptation \((P = 0.02; \text{Figure 3.4 D})\). Wojtaszewski et al. (2003) have previously reported that resting \( \text{ACC}\beta \text{ Ser}^{221} \) phosphorylation was higher in subjects with low- compared to high resting muscle glycogen stores. In that study (Wojtaszewski, et al., 2003) concentrations of creatine phosphate and adenine nucleotides in resting and exercised muscles were not altered by the glycogen manipulation leading these workers to suggest that in basal conditions at least, fuel-dependent mechanisms independent of energy status may regulate AMPK signalling. Due to insufficient muscle sample in the present investigation, high-energy phosphate contents and muscle metabolites could not be measured, but using the identical diet-exercise regimen and subjects of comparable training status, similar resting values for muscle adenine nucleotides was reported by Stellingwerff et al. (2006). However, in contrast to the findings of Wojtaszewski et al. (2003), Stellingwerff et al. (2006) found that after 20 min of cycling at the same intensity as employed in the current study, fat adaptation was associated with a lower accumulation of free ADP and AMP after 20 min of submaximal exercise compared to the high carbohydrate intervention (Stellingwerff, et al., 2006).

In conclusion, the present study used a human model of ‘dietary periodisation’ (i.e., fat adaptation followed by carbohydrate restoration) to study the interactive effects of alterations in fuel availability (i.e. muscle lipid stores) and exercise training on AMPK signalling and metabolism. It was found that, compared to an isoenergetic carbohydrate diet for 6 d, 5 d of a
high-fat diet followed by 1 d of rest and a high carbohydrate diet resulted in higher resting muscle triglyceride levels. The altered muscle fuel status at rest was associated with increased AMPKα1 and α2 activity. During exercise, FAT-adapt increased rates of whole-body fat oxidation and ‘spared’ muscle glycogen. Accordingly, the increase in ACCβ Ser^{221} phosphorylation was greater after fat adaptation compared to the high carbohydrate diet. The changes in AMPK activation observed in the present study could be due to altered fuel status (increased muscle lipid availability), a greater adaptive response when training with low muscle glycogen availability, or most likely, the interactive effects of both these factors.
Chapter Four

Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens

Adapted from: Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL, Hawley JA.

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4.1 Introduction

Training capacity (the ability to complete strenuous training sessions over days and weeks) and nutrition are highly interrelated and it is generally assumed that optimal adaptation to the demands of repeated training sessions requires a diet that can sustain muscle energy reserves (Burke, 2003; Coyle, 2000). However, evidence is accumulating to suggest that commencing endurance exercise with low muscle glycogen content increases the transcription rate of several genes and proteins involved in the training response/adaptation (Febbraio, et al., 2002; Furuyama, et al., 2003; Keller, et al., 2001; Pilegaard, et al., 2002; Steinberg, et al., 2006; Wojtaszewski, et al., 2003). Indeed, in recent years it has become evident that commencing exercise in the face of low muscle glycogen stores amplifies the activation of a number of signalling proteins, including the AMP-activated protein kinase (AMPK) and the p38 mitogen-activated protein kinase (MAPK), two enzymes with direct roles in controlling the expression and activity of several transcription factors involved in mitochondrial biogenesis and promoting training adaptation [for review see Baar and McGee (2008), Hawley et al. (2006)]. Accordingly, the notion that commencing training with low muscle glycogen levels (‘train-low’) to maximize the physiological response/adaptation to endurance training has gained popularity (Baar and McGee, 2008).

Only one study has tested the hypothesis that undertaking a training programme in which a portion of exercise sessions are deliberately commenced with low muscle glycogen may be beneficial for training adaptation and subsequent performance. In that investigation, Hansen et al. (2005) recruited 7 untrained males to undertake a 10 wk programme of leg knee extensor ‘kicking’ exercise. In an ingenious experimental design, both of the subject’s legs were trained
according to a different daily schedule but the total amount of work undertaken by each leg over the study duration was the same: subjects trained one leg twice-a-day, every second day, whereas the contra lateral leg was trained daily. Compared to the leg that performed daily training with normal glycogen reserves, the leg that commenced half of the training sessions with low muscle glycogen levels had a more pronounced increase in resting glycogen content and citrate synthase activity (Hansen, et al., 2005). Remarkably, this ‘train-low’ approach resulted in an almost two-fold greater training-induced increase in one-leg exercise time to fatigue compared to when participants commenced all training sessions in a glycogen replete state. The results of Hansen et al. (2005) demonstrate that under specific experimental conditions, training adaptation may be augmented by a relative lack of glycogen availability and this, in turn, leads to an enhancement in endurance.

While the results of that study (Hansen, et al., 2005) are intriguing, several issues currently preclude exercise scientists from making firm recommendations to athletes with respect to undertaking endurance training for performance enhancement in a low glycogen state. First, the subjects in the investigation of Hansen et al. (2005) were untrained and it is not currently known whether undertaking training sessions in a lowered glycogen state will translate into improved training adaptation and performance in already well-trained athletes. Second, the training sessions undertaken by subjects in that study were ‘clamped’ at a fixed submaximal intensity for the duration of the training programme: athletes typically periodise their programmes to incorporate a ‘hard-easy’ pattern to the overall organization of training, as well as progressive overload (Hawley and Stepto, 2001; Stellingwerff, et al., 2007a) rather than training to a fixed (submaximal) intensity. Third, the mode of training (one-legged knee
extensor kicking) and the exercise ‘performance’ task (submaximal kicking to exhaustion) bear little resemblance to the whole-body training modes and performance tasks undertaken by the majority of competitive athletes. Fourth, training schedules that induce chronically low muscle glycogen levels may increase the risk for the overtraining syndrome (Petibois, et al., 2003) and actually lead to a reduced training capacity (Achten, et al., 2004). Finally, the vast majority of athletes are reluctant to take complete rest days, and training every second day would be considered an extreme practice amongst this cohort. Accordingly, the aims of the present study were to determine the effects of undertaking selected training sessions with low muscle glycogen content on 1) training capacity and endurance performance, 2) whole-body substrate metabolism during submaximal exercise and 3) several mitochondrial enzymes and signalling proteins with putative roles in promoting training adaptation. Well-trained subjects were selected for this investigation: it was hypothesised that these athletes would already have maximised their training adaptation and that further gains would be minimal, irrespective of whether they trained with low or normal levels of muscle glycogen.

4.2 Methods

4.2.1 Subjects and preliminary testing

Eighteen endurance-trained male cyclists or triathletes volunteered to participate in this study after they were informed about the possible risks of all procedures. All subjects gave written consent. This study was approved by the RMIT University Human Research Ethics Committee. Of the eighteen subjects (Table 4.1), 14 took part in the chronic training study while 12 subjects (which included 8 subjects from the chronic study plus an additional 4 subjects who met the same criteria for age, fitness level and training history) participated in the acute phase of the
investigation (described in detail subsequently). In the 6 wk prior to commencement of the study, subjects were riding 300-500 km/wk but were not undertaking any interval training. Subjects had a history of > 3 yr of endurance-based training. One week prior to an experimental testing session, each subject undertook an incremental cycling test to exhaustion on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands). The testing protocol has been described in previous study in Chapter 3 and was originally used in Hawley and Noakes (1992). During the maximal test and the subsequently described experimental trials, subjects breathed through a Hans Rudolph two way non-rebreathing valve and mouthpiece attached to a calibrated online gas system (Parvomedics, Utah, U.S.A) interfaced to a computer, that calculated the instantaneous rates of O$_2$ consumption (VO$_2$), CO$_2$ production (VCO$_2$), minute ventilation (VE$_{STPD}$), and the respiratory exchange ratio (RER) every 15 s from conventional equations (Péronnet and Massicotte, 1991). Before each maximal test and all experimental trials, the analysers were calibrated with commercially available gases of known O$_2$ and CO$_2$ content. Peak VO$_2$ was defined as the highest O$_2$ uptake a subject attained during any 60 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final noncompleted work rate multiplied by 25 W. This value was used to determine the power output corresponding to 70% of each subject’s VO$_2$peak (63% of PPO) to be used in the subsequently described experimental trials and training sessions. The maximal test and all experimental trials were conducted under standard laboratory condition (18-22°C, 40-50% relative humidity) and subjects were fan cooled during all exercise sessions.
Table 4.1. Characteristics of the subjects that participated in the 3-wk chronic training study and the acute study. PPO, Peak sustained power output. Values are means ± SE.

<table>
<thead>
<tr>
<th>Subject characteristics</th>
<th>Age (yr)</th>
<th>Body Mass (kg)</th>
<th>VO$_{2\text{peak}}$ (L·min$^{-1}$)</th>
<th>PPO (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic study (n=14)</td>
<td>30.0 ± 1.8</td>
<td>77.7 ± 2.7</td>
<td>4.7 ± 0.1</td>
<td>382.1 ± 9.3</td>
</tr>
<tr>
<td>Acute study (n=12)</td>
<td>28.1 ± 2.0</td>
<td>77.4 ± 3.3</td>
<td>4.7 ± 0.2</td>
<td>365.5 ± 7.9</td>
</tr>
</tbody>
</table>

### 4.2.2 Experimental Design

An overview of the experimental design is shown in Figure 4.1. In brief, the subjects were divided into two groups (matched for age, VO$_2$ peak and training history) and undertook 18 laboratory training sessions during a 3 wk intervention period. The control group (HIGH) trained 6 days per week (rest on day-7) for 3 wk, alternating between 100-min steady state aerobic training (AT; ~70% VO$_2$ peak, 63% of PPO) on the first day and high intensity interval training (HIT; 8 x 5-min work bouts at maximal effort with 1-min recovery in between work bouts at ≤ 100W) the next day. In contrast, the experimental group (LOW) trained twice per day, every second day, performing the AT in the morning, followed by 1-2 hr of rest and then HIT (Figure 4.1). During the time between these two training sessions, subjects rested in the lab and were given *ad libitum* access to water. Subjects groups were designated as HIGH or LOW: HIGH completed all HIT sessions at a time when muscle glycogen levels were restored whereas LOW
undertook these HIT sessions at a time when muscle glycogen stores were partially depleted, or lower than normal. Accordingly, the 100-min AT was used in this study because it has previously been reported that endurance-trained cyclists comparable in training status to those in the current investigation utilised ~60% of their muscle glycogen content after 105 min of steady-state cycling at ~70% of individual VO$_{2\text{peak}}$ (Coyle, et al., 1986). The HIT session utilised in this study has been described in detail previously (Stepto, et al., 2001). In brief, this training session involves 8 repetitions of 5-min work bouts at ~75-80% of PPO, separated by 1 min of recovery at ≤ 100W. It has been previously reported that this workout utilizes ~50% of muscle glycogen stores when the session is commenced with normal glycogen content (~500 µmol/g dry wt.) (Stepto, et al., 2001). All the training sessions were performed using the subject’s own bicycle attached to a stationary trainer (Kinetic, MN, USA) and training intensities were monitored using PowerTap power meters (CycleOps, Madison, USA). Before every training session, the PowerTap was zeroed according to the manufacturer’s instructions. These devices have previously been shown as a valid and reliable tool to accurately monitor power output in both lab and field settings (Bertucci, et al., 2005; Gardner, et al., 2004; Paton and Hopkins, 2006). All HIT sessions in the HIGH group and all training sessions in the LOW group were performed in the laboratory under supervision of the principal investigator. Subjects in the HIGH group performed their AT at home on an indoor stationary trainer. Power output for all these sessions were recorded to ensure that the subjects trained at the prescribed intensity (63% PPO) and for the required duration (100 min).
4.2.3 Dietary control

The goal of the present study was to provide a similar dietary treatment to that received by subjects in the Hansen et al. (2005) study. A variety of methods were used to achieve this in this group of free-living subjects. Dietary intake was ‘clamped’ 24 hr prior to an experimental trial (0.21 MJ/kg BM; 8 g/kg/d and 65% of energy from CHO; 2.0 g/kg/d protein and 1.0 g/kg/d fat).
All meals and snacks were supplied with diets being individualized for food preferences and BM. Subjects received their food in pre-prepared packages and were required to keep a food checklist to note their compliance to the dietary instructions and their intake of any additional food or drinks. This method was also used during the day(s) preceding the training sessions that were chosen for examination of the acute response to exercise (see below). During the remainder of the training period, subjects were given dietary instructions by a sports dietician, including sample diets and check-lists of carbohydrate-rich foods to ensure that they were consuming 8-9 g/kg/d BM of CHO. Every 2 days, subjects were required to submit records of their dietary intake and morning BM to the sports dietician to ensure adherence to carbohydrate intake targets and to fine tune energy intake to maintain energy balance.

4.2.4 Acute responses to AT and HIT sessions

Twelve subjects (including 8 from the experiments described previously and 4 additional subjects who met the same criteria for age, VO$_{2peak}$ and training history) gave their informed consent to complete one session of AT and one session of HIT according to the training schedule for their designated group. One week prior to the commencement of a training session, the 4 additional recruits reported to the laboratory to complete preliminary testing (described previously). Subjects were rested and provided with packaged diets (described above) for the 24 hr prior to these training sessions. Subjects performed the training sessions on their own bikes attached to a stationary trainer with power meters attached (described previously). On arrival in the laboratory, a single leg was prepared for skeletal muscle biopsy sampling through two incisions 2–3 cm apart along the vastus lateralis muscle. A resting muscle sample was then taken using the percutaneous biopsy technique with suction applied while a second biopsy was obtained immediately before
the HIT. All muscle biopsies were rapidly frozen in liquid N\textsubscript{2} within ~15 s and stored at -80\textdegree C and later analysed for muscle glycogen content. In the case of the subjects who were concurrently involved in the chronic training study, these invasive sessions were performed mid-way through the 3 wk training programme (Figure 4.3).

4.2.5 Experimental trial

Forty-eight hr before the first training session and 48 hr after the last training session, subjects reported to the laboratory after a 12- to 14-h overnight fast to undertake an experimental trial comprising of a 60-min steady state ride at 70\% of VO\textsubscript{2peak} (60SS) followed by a 60-min performance trial. Skeletal muscle biopsies (described previously) were performed at rest upon subjects’ arrival in the laboratory and immediately after the 60SS. The subjects were given 10 min of rest after the resting biopsy before began the 60SS and after 10, 30 and 50 min, expired gas was collected (for 5-min) to estimate the instantaneous rates of substrate oxidation. Fifteen min after the completion of 60SS, the subjects began the performance ride. Endurance performance was determined as the average power maintained (W/kg BM) during the 60-min period.

4.2.6 Analytical Procedures

Rates of whole body fat oxidation.

Rates of whole body fat oxidation (g/min) were calculated from the RER data collected for 5 min at 10, 30 and 50 min of the 60SS. The calculations were made from VCO\textsubscript{2} and VO\textsubscript{2} measurements, assuming a nonprotein RER value, according to the following equation (Péronnet and Massicotte, 1991).
Fat oxidation = 1.695 VO₂ - 1.701 VCO₂

Rates of fatty acid oxidation (µmol/kg/min) were determined by converting the rate of triglycerol oxidation (g/min) to its molar equivalent assuming the average molecular weight of human triglycerol to be 855.3 g/mol and multiplying the molar rate of triglycerol oxidation by 3, because each molecule contains three molecules of fatty acids. Total fat oxidation during the 60SS was estimated by calculating the area under the oxidation versus time curves for each subject.

Muscle glycogen concentration

Approximately 10-15 mg of muscle was freeze-dried and powdered with all visible blood and connective tissue removed under magnification. The freeze-dried muscle sample was then incubated in 250 µL of 2 M hydrochloric acid at 100 °C for 2 hr before being neutralized with 750 µL of 0.67 M sodium hydroxide. Glycogen concentration was determined via enzymatic analyses (50 mM Tris, 25 mM HCL, 1 mM MgCl₂, 0.5 mM DTT, 0.3 mM ATP, 0.05 mM NADP and 1 U/ml HK, 0.1 U/ml G-6-PDH) with glucose standards by fluorometric detection and was expressed as micromoles of glycogen/g dry wt.

Citrate synthase and β-hydroxyacyl-CoA dehydrogenase activity.

Freeze-dried, powdered muscle (2-3 mg) was homogenised in 100 mM potassium phosphate buffer (pH 7.3, 1:400 dilution), and citrate synthase was assayed spectrophotometrically at 25°C by the reduction of DNTB, as published previously (Srere, 1969) with slight modifications (Bruce, et al., 2003). β-hydroxyacyl-CoA dehydrogenase (β-HAD) activity was assayed spectrophotometrically at 25°C using the same homogenates, based on the disappearance of reduced NADH (Lowry and Passonneau, 1972).
Western blotting

Approximately 30 mg of wet muscle was homogenised (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and the homogenates were then centrifuged at 20,000 g for 30 min at 4ºC. The supernatant was aliquoted and stored at -80ºC until further analysis. The total protein concentration in the aliquots was determined by the bicinchoninic acid method (Pierce, IL, USA).

Muscle lysates containing 60 µg (pAMPK Thr172, Total AMPK and PGC-1α) and 30 µg [Cytochrome C oxidase subunit II and IV (COX II and COX IV respectively)] of total protein were electrophoresed on 10% (pAMPK Thr172, Total AMPK and PGC-1α) and 14% (COX II and COX IV) SDS-PAGE and detected by immunoblotting with antibodies specific for PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA; sc13067), α-tubulin (Cell Signalling, Danvers, MA; #2144), pAMPK Thr172 and Total AMPKα (a gift from Professor Bruce Kemp), COX II (Mitosciences, Oregon; MS 405) and COX IV (Mitosciences, Oregon; MS 407). Since most analyses required completion of multiple gels due to the large number of samples, an identical internal standardised human skeletal muscle sample, designated “Control”, was used in all gels to account for variability between exposures of different membranes. PGC-1α, COX II and COX IV protein content was expressed relative to total α-tubulin, whereas pAMPK Thr172 was expressed relative to total AMPK. The immunoreactive proteins were detected with enhanced chemiluminescence (Amersham Biosciences) on a BioRad Chemidoc XRS system (BioRad, CA) and quantified by densitometry (Quantity one, BioRad, CA).
Mitochondrial DNA determination

Mitochondrial DNA (mtDNA) was quantified by real-time PCR using methods described previously (Menshikova, et al., 2005), with modification and optimisation to allow multiplex PCR. Briefly, total mitochondrial and nuclear DNA was isolated from ~20 mg of snap-frozen muscle tissue using a commercially available kit (Qiagen, Victoria, Australia), and the concentration of each sample was determined using a Nanodrop ND-1000 spectrophotometer (ThermoFisher Scientific, DE, USA). Ten ng of DNA from each sample was amplified in a BioRad iCycler PCR detection system using BioRad iQ Supermix (BioRad, NSW, Australia), 900 nM of forward and reverse primers for mtDNA and β-globin as a housekeeping gene (Sigma Genosys, NSW, Australia), and 225 nM of FAM-labelled Taqman probe for mtDNA and VIC-labelled probe for β-globin (Applied Biosystems, Victoria, Australia). Reaction volume was 25 µl, and primer and probe sequences were as previously reported (Menshikova, et al., 2005). Data were quantified using the delta-Ct method to quantitate fold changes in mtDNA compared to pre-training values.

Statistical Analysis

Treatment effects were analysed using two-factor (treatment and time) repeated measure analysis of variance (ANOVA) with post-hoc analysis performed using the Holm-Sidak method. Data were analysed using SigmaStat 3.1.1 (Systat Software, Inc., CA) and all values are expressed as means ± standard error (SE), with significance reported as p<0.05.
4.3 Results

4.3.1 Muscle glycogen concentration before AT and HIT training sessions

Figure 4.2 displays muscle glycogen content before AT and HIT in the subgroup of 12 subjects who participated in the acute phase of the study. As intended, muscle glycogen stores in HIGH were similar before both AT and HIT (~400 μmol/g dry wt.). In LOW, however, muscle glycogen stores were significantly reduced after AT ($P < 0.05$, Figure 4.2) such that LOW commenced the HIT with ~50 % less glycogen than before the AT.

![Figure 4.2](image-url)

**Figure 4.2.** Muscle glycogen concentration before aerobic training (AT) and before high intensity interval training (HIT) during the acute training study. * Significantly different from Pre-AT ($P < 0.05$). Values are mean ± SE; $n \geq 6$/group.
4.3.2 Training intensity during HIT

During week 1 (HIT 1-3), LOW trained at a significantly lower average percentage of PPO compared to HIGH ($P < 0.05$, Figure 4.3). During the second week (HIT sessions 4-6) of intervention, there was a strong trend for the average training intensity to be lower ($P = 0.06$, Figure 4.3) but by the third week (HIT sessions 7-9), the average training intensities between LOW and HIGH were not different (Figure 4.3).

Figure 4.3. Training intensity expressed as percentage of peak power output (PPO) during HIT sessions. * Significantly different between HIGH and LOW ($P < 0.05$); $n = 7$ / group.
4.3.3 Resting muscle glycogen concentration

After 3 wk of training, resting muscle glycogen concentration increased significantly from 412 ± 51 μmol/g dry wt. to 577 ± 34 μmol/g dry wt. ($P < 0.05$, Figure 4.4) in LOW. While glycogen levels increased in HIGH, such changes failed to reach statistical significance.

Figure 4.4  Resting muscle glycogen concentration before and after the 3-week training intervention. * Significantly different from Pre-training ($P < 0.05$). Values are mean ± SE; $n \geq 6$/group.
4.3.4 Fat oxidation during 60-min steady-state ride

Estimated rates of fat oxidation during the 60SS after 10, 30 and 50 min of the ride are shown in Figure 4.5. Fat oxidation during the 60SS, measured as the total area under the fat oxidation vs. time curve (AUC) showed a strong trend ($P = 0.051$) to be greater in LOW after the 3 wk training period ($1698 \pm 174$ vs. $1261 \pm 247 \, \mu\text{mol/kg/60 min}$). The AUC measured as the last 40 min of the 60SS was significantly greater in LOW after training ($1220 \pm 100$ vs. $867 \pm 166 \, \mu\text{mol/kg/40 min}$, $P < 0.05$).

![Figure 4.5](image)

Figure 4.5 Rates of whole-body fat oxidation at 10, 30 and 50 min before (Pre) and after (Post) the 3-week training intervention. * Significantly different from Pre-training ($P < 0.05$). Values are mean ± SE; n = 7/group.
4.3.5 Citrate Synthase and $\beta$-HAD activity

Maximal citrate synthase activity in LOW increased from $45 \pm 2$ mmol/kg/min dry wt. to $54 \pm 1$ mmol/kg/min dry wt. (Figure 4.6A; $P < 0.05$) after the 3 wk training period such that the post-training maximal activity in LOW was significantly higher than the post-training maximal activity in HIGH (Figure 4.6A; $P < 0.05$). The maximal citrate synthase activity remained unchanged in HIGH (39 $\pm$ 5 mmol/kg/min dry wt. to 43 $\pm$ 3 mmol/kg/min dry wt., Figure 4.6A) after the 3 wk training period.

$\beta$-HAD activity increased in LOW (18 $\pm$ 2 mmol/kg/min dry wt. to 23 $\pm$ 2 mmol/kg/min dry wt.; $P < 0.05$; Figure 4.6B) such that the post-training maximal activity in LOW was significantly higher than the similar time point in HIGH (Figure 4.6B; $P < 0.05$). The maximal $\beta$-HAD activity remained unchanged in HIGH after the 3 wk training period (16 $\pm$ 1 mmol/kg/min dry wt. to 17 $\pm$ 1 mmol/kg/min dry wt.).

4.3.6 Cytochrome c oxidase subunits II and IV

The total protein content of COX II (relative to total $\alpha$-tubulin content) remained unchanged after the 3-wk training intervention in both groups (Figure 4.6C). However, the total protein content of COX IV (relative to total $\alpha$-tubulin content) was significantly higher only in LOW after the 3-wk training intervention ($P < 0.05$, Figure 4.6D).
Figure 4.6  (A) Maximal citrate synthase activity, (B) Maximal $\beta$-hydroxyacyl-CoA-dehydrogenase activity, (C) Total protein content of cytochrome c oxidase subunit II, and (D) Total protein content of cytochrome c oxidase subunit IV before (Pre) and after (Post) the 3-week training programme. * Significantly different compared to LOW-Pre-training ($P < 0.05$). # Significantly different compared to HIGH-Post-training ($P < 0.05$). Values are mean ± SE; n = 7/group.
4.3.7 Mitochondrial DNA

There was no effect of either training programme on skeletal muscle mtDNA content after the 3 wk training interventions (Figure 4.7).

![Bar chart showing mtDNA content](chart.png)

**Figure 4.7** Mitochondrial DNA content before (Pre) and after (Post) the 3-week training programme. Values are mean ± SE. n = 7/group.

4.3.8 pAMPK\textsuperscript{Thr172} and PGC1α

Phosphorylation of AMPK at threonine-172 measured in skeletal muscle biopsy samples was unchanged after 1 hr of submaximal cycling both before and after training (Figure 4.8A).

Total protein content of PGC-1α (relative to total α-tubulin) content was also unchanged after the 3-wk training intervention (Figure 4.8B).
Figure 4.8  Representative western blots for (A) phosphorylation of AMPK at threonine-172 and total AMPK at rest and after exercise, before (Pre) and after (Post) 3-week training intervention, and (B) Resting total peroxisome proliferator-activated receptor gamma coactivators-1α (PGC-1α) and α-tubulin protein content before (Pre) and after (Post) 3-wk training intervention. Exerc, Exercise. Control, standardised protein sample loaded to account for variation between membranes.
4.3.9 Endurance performance

Figure 4.9 displays the average power output (corrected for BM) maintained during the 60-min performance trial before and after the 3-wk training intervention, along with individual changes for each subject. After the 3 wk training intervention, performance during the 60-min time trial was significantly higher for both groups (12.2 ± 2.3 % vs. 10.2 ± 3.1 % for LOW and HIGH respectively, $P < 0.01$; Figure 4.9). The magnitude of increase in performance was not different between groups.

Δ = 10.2 ± 3.1 %  
Δ = 12.2 ± 2.3 %

![Graph showing power output before and after training](image)

Figure 4.9 Cycling performance as measured by the average power output (W) maintained during the 60 min time trial before (Pre) and after (Post) 3-week training intervention. † Significantly different from Pre ($p<0.01$). Values are mean ± SE; n = 7/group.
4.4 Discussion

The novel findings of the present study were that in skeletal muscle of trained individuals 1) resting glycogen content, 2) the maximal activities of citrate synthase and $\beta$-hydroxyacyl-CoA dehydrogenase, 3) the content of the electron transport chain component cytochrome C oxidase subunit IV, and 4) rates of whole-body fat oxidation during submaximal exercise were enhanced to a greater extent by training twice every second day compared to training daily for 3 weeks. These findings are in direct contrast to the original research hypothesis of this study, namely that well-trained athletes would have maximised their training adaptation, and that further gains would be minimal, independent of whether they trained daily or twice every second day. Nevertheless, despite metabolic and enzymatic changes resulting in an enhanced training adaptation profile after twice every second day ‘low glycogen’ training, this study was unable to detect a clear advantage to endurance performance compared to when subjects undertook daily workouts with normal glycogen stores.

The present investigation was undertaken to further examine the hypothesis that commencing exercise sessions with low muscle glycogen levels would result in a more pronounced training adaptation compared to training in a normal glycogen condition (Febbraio, et al., 2002; Hansen, et al., 2005). Accordingly, subjects performed either two exercise bouts on the same day (100 min of submaximal cycling followed by 1-2 hr rest and then an intense interval training session) or the same training sessions separated by 24 hr. The former training protocol (training twice every second day) resulted in a marked decrease in muscle glycogen concentration after the first exercise session, such that subjects commenced the second bout of training with significantly lower muscle glycogen content than before the first session of the day (Figure 4.2). In contrast,
when subjects performed the prolonged cycling bout and had 24 hr recovery, they began the intense interval training bouts with normal glycogen stores (Figure 4.2). While the term ‘train low, compete high’ has been used to describe the twice every second day protocol (Hansen, et al., 2005), a fact often overlooked is that subjects in that study (and the present investigation) only performed 50% of their training sessions with low muscle glycogen content.

The present study chose to incorporate high intensity interval training sessions in combination with prolonged, steady-state aerobic riding as competitive cyclists typically employ such workouts in their race preparation [for review see (Hawley and Stepto, 2001)]. Previous studies have consistently demonstrated substantial performance enhancements after just 3 weeks of HIT in already well trained athletes (Lindsay, et al., 1996; Stepto, et al., 1999; Westgarth-Taylor, et al., 1997; Weston, et al., 1997). Furthermore, previous study has also shown that when subjects commence HIT sessions with normal glycogen levels (~450-500 μmol/g dry wt.), they deplete ~50% of resting stores (Stepto, et al., 2001). Hence, it was not surprised to observe that when subjects performed self-selected HIT sessions at maximal effort following 100 min of cycling, at a time when glycogen stores were already 50% reduced, relative power output (the percentage of PPO sustained for the 8 x 5 min work bouts) was significantly lower compared to when the same sessions were undertaken in the glycogen replete state (Figure 4.3). Of note was that while self-selected maximal power output was significantly lower for the first 6 HIT sessions (i.e., the first two weeks of the training programme), during the last week of training there were no differences in average power output whether or not subjects commenced the workouts with low or normal glycogen stores (Figure 4.3). The subjects are believed to have produced maximal efforts for all HIT sessions. Indeed, in order to ensure compliance, attractive financial incentives were provided
to the subject from each training group who performed the most overall work (when work was corrected for PPO and BM).

Resting muscle glycogen stores are typically increased by 20-30% when untrained subjects complete short term (i.e. < 6 weeks) endurance training programmes [for review see Hargreaves (1997)]. Resting muscle glycogen levels in endurance-trained subjects are consistently elevated (i.e. 450-550 μmol/g dry wt.) above untrained individuals (Hawley, et al., 1997). Hence, a surprising finding in the present study was that training twice every second day resulted in a further increase in muscle glycogen stores (Figure 4.4). These results are in agreement with those of Hansen et al. (2005) who only found elevated resting muscle glycogen content after their ‘train-low’ intervention.

In order to determine whether the training adaptation could be amplified by training twice every second day, indirect markers of tricarboxylic acid cycle flux (citrate synthase), β oxidation (β-HAD) and electron transport chain activity (cytochrome c oxidase subunits) were measured. The increase in the activity/content of these mitochondrial enzymes only reached statistical significance in the group that trained twice every second day (Figure 4.6). It may seem surprising that 3 weeks of training is sufficient to elevate maximal enzyme activities in already trained subjects. However, previous study has also observed increases in β-HAD activity after just 5 days of intense training incorporating two sessions of HIT in well-trained athletes ingesting a low-carbohydrate, high-fat diet (Cameron-Smith, et al., 2003). The potential mechanism for the increased mitochondrial enzyme activity after training twice every second day is hard to define, and it is possible that exercising with low glycogen stores could promote training adaptations.
through perturbation in homeostasis (i.e. increased systemic factors) and not directly through low glycogen availability *per se* (Baar and McGee, 2008). Although catecholamine levels were not measured during training in the present study, Hansen et al. (2005) have reported that the catecholamine response to exercise performed with low muscle glycogen levels are higher than when exercise undertaken with normal glycogen stores, demonstrating a higher stress response.

In contrast to the increases in mitochondrial enzyme activities induced by the twice every second day training regimen, mitochondrial DNA content and PGC-1α protein content (Figure 4.8B) were unchanged in response to both training interventions. Mitochondrial DNA is a marker of mitochondrial volume and is increased in trained compared to untrained individuals (Puntschart, et al., 1995). Furthermore, mtDNA typically parallels increases in mitochondrial density, although not necessarily by the same magnitude as mitochondrial volume (Puntschart, et al., 1995). Consequently the ~20% increases in maximal enzyme activity observed after training twice every second day could coincide with a small increase in mitochondrial volume that is not detectable through mtDNA analysis (Figure 4.7). PGC-1α plays a role in regulating the expression of genes encoding mitochondrial proteins in skeletal muscle (Hood, et al., 2006). Importantly, PGC-1α can also co-activate the transcription factor nuclear respiratory factor-1 to regulate a nuclear encoded protein (mitochondrial transcription factor A or Tfam) that controls mtDNA replication and transcription (Joseph, et al., 2006). Although no previous study has examined the effect of twice every second day training on PGC-1α protein content, Mortensen et al. (2007) have previously reported that this training regimen does not increase the mRNA expression of the PGC-1α family of transcriptional coactivators. While an acute bout of high intensity training increases PGC-1α content to a greater extent than low intensity exercise
(Terada, et al., 2005), and chronic short duration interval training elevates PGC-1α to the same extent as more traditional endurance workouts (Burgomaster, et al., 2008), it is unlikely that the changes in power output observed between the twice every second day and once-a-day interval training sessions were large enough to increase PCG-1α protein levels. In addition, PGC-1α content was measured, but not transcriptional co-activity, it cannot be ascertain as to whether or not completion of high intensity training in the LOW group transiently increased PGC-1α activity, thereby contributing to the changes in mitochondrial enzymes measured.

Analogous to PGC-1α, the AMPK has been shown to have important regulatory roles in both the responses to an acute bout of exercise and also chronic training adaptations [for review see Aschenbach et al. (2004)]. The AMPK is an important sensor of decreased energy charge in cells and subsequently acts to increase catabolic reactions and decrease anabolic reactions, one of which is the direct phosphorylation and subsequent increased transcriptional co-activity of PGC-1α (Jager, et al., 2007). In this regard, Wojtaszewski et al. (2003) have previously reported that AMPK activity in resting human muscle and the degree of activation during an acute exercise bout are dependent on the fuel status of the muscle cells (i.e., AMPK activity is elevated in muscle with low glycogen stores). In the present investigation AMPK phosphorylation and total AMPK protein content was similar before and after both training interventions (Figure 4.8A). Thus, the results of the present study indicates that the 3 week training programme (in which subjects performed 50% of their training sessions with low starting muscle glycogen content) was insufficient to increase AMPK protein levels and/or activation in already well-trained individuals. This finding is in agreement with Clark et al. (2004) who previously reported that 3 weeks of
intensified training in well-trained athletes does not alter AMPK signalling in skeletal muscle in response to a submaximal exercise bout.

In order to assess the effect of the different training protocols on the metabolic responses to submaximal exercise, subjects performed 60 min of steady-state cycling pre- and post intervention. In accord with the elevated $\beta$-HAD activity after the twice every second day training programme, the present study also reported a robust increase in rates of whole-body fat oxidation compared to once-a-day training (Figure 4.5). Yet, despite creating metabolic conditions that should, in theory, enhance endurance capacity, training twice every second day failed to increase the performance of a 1 hour time-trial (a performance measure similar to road cycling time-trials conducted at major championships and Tours) undertaken after 1 hr of submaximal cycling, to a greater extent than one-a-day training. Previous studies (Burke, et al., 2000; Burke, et al., 2002; Carey, et al., 2001; Havemann, et al., 2006; Helge, 2000; Stellingwerff, et al., 2006) have also repeatedly demonstrated the ability of well-trained subjects to further improve their ability to oxidise fat and ‘spare’ carbohydrate after short-term (< 7 days) dietary/training periodisation. Part of the reason why nutrient/training interventions that enhance fat-oxidative capabilities do not confer concomitant performance benefits is that the observed ‘carbohydrate sparing’ is more likely to be an impairment of carbohydrate oxidation due to a down-regulation of the multi-enzyme complex pyruvate dehydrogenase (PDH) (Stellingwerff, et al., 2006). Unfortunately, the muscle tissue in the present study was insufficient to assess PDH activity, but such a measure would provide useful mechanistic insight into training response/adaptation in future studies that manipulate the nutrient/training environment.
The finding of no difference in a whole-body endurance performance task contrasts that of Hansen et al. (2005) who reported that training twice every second day resulted in superior endurance capacity compared to training daily. Several major differences in the training protocols between the current study and that of Hansen et al. (2005) are likely to be responsible for the contrasting effects on performance. First, whole-body exercise (cycling) versus a one-legged kicking model was chosen as the training mode. Second, intense interval training sessions were incorporated into the 3 week training programmes. Third, subjects were allowed to self-select the highest sustainable power output during the HIT sessions, whereas Hansen et al. (2005) ‘clamped’ the training intensity. Fourth, athletes who had completed a base of aerobic training before entering the study were utilised, compared to previously healthy, but untrained subjects chosen by Hansen et al. (2005). Notwithstanding such differences, one might offer an alternative perspective on the results from the present investigation. Namely, despite compromised high-intensity training capacity, the twice every second day training regimen elicited a comparable increase in endurance performance to that attained after training every day. Thus for an athlete unable to train daily but who can perform two workouts in close proximity, with the second session performed under conditions of low starting muscle glycogen, ‘train-low’ may offer a time efficient method of maintaining training adaptations and performance.

In conclusion, compared to training daily, training twice every second day compromised high-intensity training capacity but augmented selected markers of training adaptation (i.e., resting muscle glycogen content, the maximal activities of several mitochondrial enzymes and the protein content of COX IV). However, despite creating conditions that, in theory, should enhance endurance performance capacity, performance of a one hour time-trial undertaken after a 60 min
steady-state ride was similar after daily or twice every second day training. Further studies will be needed to determine whether low muscle glycogen stores per se or perturbation in systemic or other intramuscular factors are responsible for the amplified training response observed after twice every second day versus daily training schedules.
Chapter Five

Acute signalling responses to intense endurance training commenced with low or normal muscle glycogen

Adapted from: Yeo WK, McGee SL, Carey AL, Paton CD, Garnham AP, Hargreaves M, Hawley JA. (in preparation)
5.1 Introduction

Substrate availability is a potent modulator of skeletal muscle training adaptation (Coyle, 2000; Hansen, et al., 2005; Hawley, et al., 2006). In the previous study described in Chapter 4, it was shown that well-trained endurance athletes who undertook a 3-wk training programme in which high-intensity interval training sessions were commenced with low muscle glycogen concentration increased selected markers of endurance training adaptation to a greater extent than athletes who began all training sessions with normal glycogen levels. These adaptations included an elevated resting muscle glycogen concentration, increases in the maximal activities of citrate synthase and β-hydroxyacyl-CoA dehydrogenase and the total protein content of cytochrome c oxidase subunit IV, and enhanced rates of whole-body fat-oxidation during submaximal exercise.

Chronic training adaptations are thought to be the result of the cumulative effects of repeated acute bouts of exercise (Hansen, et al., 2005; Hawley, et al., 2006; Pilegaard, et al., 2000; Widegren, et al., 2001). As such, one potential mechanism that might underlie the greater adaptation seen after training with low muscle glycogen availability is the acute myocellular stress associated with this state which may augment the activation of several protein kinases with roles in mitochondrial biogenesis. These include the AMPK (Steinberg, et al., 2006; Wojtaszewski, et al., 2003) and the p38 MAPK (Chan, et al., 2004). Accordingly, the purpose of the present study was to investigate the acute signalling responses to a single session of HIT undertaken with either low or normal muscle glycogen concentration. It was hypothesised that performing intense endurance exercise in the face of low muscle glycogen concentration would result in increased activation of AMPK and p38 MAPK and some of their downstream substrates.
5.2 Methods

5.2.1. Subjects and Preliminary Testing

Twelve endurance-trained male cyclists or triathletes volunteered to participate in this study. They gave their written consent after they were informed about the possible risks of all procedures. This study was approved by the RMIT University Human Research Ethics Committee. One week prior to the commencement of the study, each subject undertook an incremental cycling test to exhaustion on an electromagnetically braked cycle ergometer (Lode, Groningen, The Netherlands). The results from this test (peak $O_2$ uptake and peak sustained power output) were then used to determine the power output corresponding to 70% of each subject’s $VO_{2peak}$ (63% of PPO) to be used in the subsequently described training sessions. The testing protocol (Hawley and Noakes, 1992) and the equipment used for this test have been described in detail in previous chapter in this thesis (Chapter 3). The maximal test and all experimental trials were conducted under standard laboratory condition (18-22°C, 40-50% relative humidity) and subjects were fan cooled during all training sessions.

Table 5.1. Characteristics of the subjects that participated in the HIGH and LOW group. PPO, Peak sustained power output. Values are means ± SE; n = 6/group.

<table>
<thead>
<tr>
<th></th>
<th>Age (yr)</th>
<th>BM (kg)</th>
<th>$VO_{2peak}$ (L/min)</th>
<th>$VO_{2peak}$ (mL/kg/min)</th>
<th>PPO (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIGH</td>
<td>29.8 ± 2.7</td>
<td>77.5 ± 4.8</td>
<td>4.7 ± 0.2</td>
<td>61.2 ± 1.4</td>
<td>370.1 ± 12.9</td>
</tr>
<tr>
<td>LOW</td>
<td>26.3 ± 2.9</td>
<td>77.1 ± 4.9</td>
<td>4.7 ± 0.2</td>
<td>61.9 ± 2.2</td>
<td>360.9 ± 10.0</td>
</tr>
</tbody>
</table>
5.2.2. Experimental Design

An overview of the experimental design is shown in Figure 5.1. In brief, the subjects were divided into two groups matched for age, VO$_{2\text{peak}}$ and training history. They then undertook two different laboratory training protocols according to the schedule of their respective groups. One group (HIGH) performed a 100-min steady state ride (AT) at ~70% VO$_{2\text{peak}}$ (63% of PPO) on the first day and high intensity interval training (HIT; 8 x 5-min work bouts at self selected maximal effort with 1-min recovery in between work bouts at $\leq 100W$) 24 hr later. In contrast, the other group (LOW) performed both training sessions on the same day, performing the AT in the morning (0800 hr), followed by HIT after 1-2 hr of rest (Figure 5.1). During the time between these two training sessions, subjects in the LOW group rested in the lab and were given *ad libitum* access to water. The training sessions and equipment set up have been described in detail in previous chapter in this thesis (Chapter 4). All training sessions were performed in the laboratory under the supervision of the principal investigator and power output for all these sessions were recorded using PowerTap power meters (CycleOps). Skeletal muscle biopsies from the vastus lateralis were taken immediately before and after the HIT sessions (Figure 5.1). The muscle biopsy procedure has been explained in detail in previous chapters in this thesis (Chapters 3 and 4). All muscle samples were rapidly frozen in liquid N$_2$ within ~15 s and stored at -80°C until further analysis.

5.2.3. Dietary and Training Control

Twenty four hr prior to all training sessions, subjects’ dietary intake were ‘clamped’ (0.21 MJ/kg BM; 8 g/kg/d and 65% of energy from CHO; 2.0 g/kg/d protein and 1.0 g/kg/d fat). All meals and snacks were supplied with diets being individualized for food preferences and body mass
Subjects received their food in pre-prepared packages and were required to keep a food checklist to note their compliance with the dietary instructions and their intake of any additional food or drinks. Empty bags and containers were also brought back to the lab to confirm the consumption of the food prescribed. Subjects refrained from exercise the day prior to an experiment.

**Figure 5.1** Overview of the study design and experimental trial. AT, Aerobic training. HIT, High intensity interval training. †, Muscle biopsy.

**5.2.4. Analytical Procedures**

**Muscle Glycogen Concentration**

Muscle glycogen concentration was analysed as described in previous chapters in this thesis (Chapters 3 and 4). In brief, approximately 10-15 mg of muscle was freeze-dried and powdered with all visible blood and connective tissue removed under magnification. The freeze-dried
muscle sample was then extracted and glycogen concentration was determined via enzymatic analyses.

Nuclear Fractionation and Whole Cell Extraction

Approximately 70-80 mg of wet muscle were cut and divided into two portions. The first portion (35-40 mg) of the wet muscle was homogenised in ice cold buffer A (250 mM sucrose, 10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl₂, 1 mM DTT, PIC [2 µL per 40 mg] and 1 mM PMSF) before being spun for 5 min at 500 x g at 4°C. The supernatant was removed and the pellets were then resuspended in ice cold buffer B (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, PIC [2 µL per 40 mg], 50 mM NaF, 50 mM MgCl₂, 1 mM DTT, 1 mM PMSF and 5 mM Na pyrophosphate) for 10 min. The lysates were then spun again for 5 min at 3000 x g at 4°C and the supernatant representing the nuclear fraction was aliquoted and stored at -80°C until further analysis.

The second portion (35-40 mg) of wet muscle was homogenised (50 mM Tris HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 50 mM NaF, 5 mM Na pyrophosphate, 10% glycerol, 1% Triton X-100, 10 µg/ml trypsin inhibitor, 2 µg/ml aprotinin, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 20,000 x g for 30 min at 4°C. The supernatant representing the whole cell lysates was aliquoted and stored at -80°C until further analysis. Total protein concentration in both the nuclear and whole cell lysates were determined by the bicinchoninic acid method (Pierce, IL, USA).
Western Blotting

Muscle lysates containing 60 μg (p-AMPK\textsuperscript{Thr172}, total AMPK, p-p38 MAPK\textsuperscript{Thr180/Tyr182}, total p38 MAPK, p-CAMKII\textsuperscript{Thr286}, PGC-1α, α-tubulin) and 50 μg (nuclear HDAC5, total HDAC5, p-CREB\textsuperscript{Ser133} and p-ATF2\textsuperscript{Thr69/71}) of total protein were electrophoresed on 10% (p-AMPK\textsuperscript{Thr172}, total AMPK, p-p38 MAPK\textsuperscript{Thr180/Tyr182}, total p38 MAPK, p-CAMKII\textsuperscript{Thr286}, PGC-1α, α-tubulin) and 5-14% (nuclear HDAC5, total HDAC5, p-CREB\textsuperscript{Ser133} and p-ATF2\textsuperscript{Thr69/71}) SDS-PAGE and detected by immunoblotting with antibodies specific for pAMPK\textsuperscript{Thr172}, total AMPKα (gifts from Professor Bruce Kemp), p-p38\textsuperscript{Thr180/Tyr182} MAPK, total p38 MAPK, nuclear HDAC5, total HDAC5, p-CREB\textsuperscript{Ser133}, p-ATF2\textsuperscript{Thr69/71}, p-CAMKII\textsuperscript{Thr286}, α-tubulin (Cell Signalling, Danvers, MA) and PGC-1α (Santa Cruz Biotechnology, Santa Cruz, CA; sc13067). An internal control (standardised human skeletal muscle sample) was used in all gels to account for variability between exposures of different membranes. The immunoreactive proteins were detected with enhanced chemiluminescence (GE Healthcare) on a BioRad Chemidoc XRS system (BioRad, CA) and quantified by densitometry (Quantity one, BioRad, CA). Phosphorylation of AMPK\textsuperscript{Thr172} and p38\textsuperscript{Thr180/Tyr182} MAPK were expressed relative to their respective total protein concentrations while p-CAMKII\textsuperscript{Thr286} and PGC-1α was expressed relative to standard control α-tubulin.

5.2.5. Statistical Analysis

Treatment effects were analysed using two-factor (treatment and time) repeated measure analysis of variance (ANOVA) with post-hoc analysis performed using the Holm-Sidak method. Data were analysed using SigmaStat 3.1.1 (Systat Software, Inc., CA) and all values are expressed as means ± standard error (SE), with significance reported as \( P < 0.05 \).
5.3 RESULTS

5.3.1 Muscle glycogen concentrations

Figure 5.2 displays muscle glycogen content before and after HIT session. There was no significant interaction between the two factors of treatment and time. There were significant main effects of both treatment and time on muscle glycogen content. As would be expected, muscle glycogen concentration was significantly higher in HIGH compared to LOW before the HIT (390 ± 28 vs. 256 ± 67 µmol/g dry wt.). After HIT, glycogen levels were reduced significantly in both groups ($P < 0.05$) such that HIGH is significantly elevated compared with LOW (229 ± 29 vs. 124 ± 41 µmol/g dry wt.; $P < 0.05$).

![Figure 5.2 Muscle glycogen concentration before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. #, Significantly different at $P < 0.05$. Values are mean ± SE; $n \geq 5$/group.](image-url)

Figure 5.2 Muscle glycogen concentration before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. #, Significantly different at $P < 0.05$. Values are mean ± SE; $n \geq 5$/group.
5.3.2 **Self selected training intensity during HIT**

Self-selected average power output during the HIT was significantly greater in HIGH compared with LOW (308 ± 14 vs. 270 ± 13 W, or 83 ± 2 vs. 75 ± 3% PPO; \( P < 0.05 \); Figure 5.3).

![Figure 5.3](image)

**Figure 5.3** Self-selected average power output during the HIT session. #, Significantly higher than LOW. Values are mean ± SE; \( n = 6 \)/group.

5.3.3 **p-AMPK\(^{Thr172}\) and p-p38 MAPK\(^{Thr180/Tyr182}\)**

Figure 5.4A displays p-AMPK\(^{Thr172}\) (relative to total AMPK) before and after HIT. There was no significant interaction between the two factors of treatment and time. There were significant main effects of treatment and time on p-AMPK\(^{Thr172}\). p-AMPK\(^{Thr172}\) was similar before the commencement of the HIT in both HIGH and LOW but increased significantly in both groups after HIT (HIGH, 3.5 ± 0.1 to 5.4 ± 0.5 vs. LOW, 3.8 ± 0.3 to 7.5 ± 1.1 arbitrary units; \( P < 0.05 \)) such that the phosphorylation state was higher in LOW compared to HIGH (\( P < 0.05 \)).

Figure 5.4B displays p-p38\(^{Thr180/Tyr182}\) (relative to total p38 MAPK) before and after HIT. There was no interaction between the two factors of treatment and time. Nor were there any significant main effects for treatment and time on p-p38\(^{Thr180/Tyr182}\) (Figure 5.4B).
Figure 5.4  Phosphorylation of (A) AMPK at threonine 172 (p-AMPK^Thr172) and (B) p38 MAPK before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. # Significantly different at $P < 0.05$. Values are mean ± SE; n = 6/group.
5.3.4 \( p\text{-CAMKII}^{Thr286} \)

Figure 5.5 displays \( p\text{-CAMKII}^{Thr286} \) (relative to \( \alpha\)-tubulin) before and after HIT. There was no significant interaction between the two factors of treatment and time. There was significant main effect of time on \( p\text{-CAMKII}^{Thr286} \) (\( \beta_m \)) but no significant main effect of treatment was observed. \( p\text{-CAMKII}^{Thr286} \) (\( \beta_m \)) was similar before HIT in both groups but was only increased significantly in HIGH after the exercise bout (\( P < 0.05 \); Figure 5.5). However, there were no significant main effects of treatment or time on \( p\text{-CAMKII}^{Thr286} \) (\( \delta/\gamma \)) or when all 3 of the \( p\text{-CAMKII}^{Thr286} \) isoforms were quantified collectively.

Figure 5.5  Phosphorylation of CAMKII (\( \beta_m \)) at threonine 286 (\( p\text{-CAMKII}^{Thr286} \)) before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. #, Significantly different at \( P < 0.05 \). Values are mean ± SE; \( n = 6 \)/group.
5.3.5 HDAC5, p-CREB$_{\text{Ser}133}$ and p-ATF2$_{\text{Thr}69/71}$

There was no significant interaction between treatment and time. Neither were there any significant main effects of treatment nor time on total or nuclear HDAC5 (Figure 5.6A), p-CREB$_{\text{Ser}133}$ (Figure 5.6B) and p-ATF2$_{\text{Thr}69/71}$ (Figure 5.6C) in response to a single bout of HIT.

Figure 5.6 (A) Nuclear concentration of HDAC5, (B) Phosphorylation of cyclic AMP response element binding protein (p-CREB), and (C) Phosphorylation of activating transcription factor 2 (p-ATF2) before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. Values are mean ± SE; n = 6/group.
5.3.6  PGC-1α

There was no interaction between the two factors of treatment and time. Neither were there any significant main effects of treatment nor time on total PGC-1α protein expression with HIT (Figure 5.7).

![PGC-1α and α-tubulin expression](image)

**Figure 5.7**  Total PGC-1α protein expression before (Pre) and after (Post) high intensity interval training for groups that performed HIT with normal (HIGH) versus low (LOW) muscle glycogen concentration. Values are mean ± SE; n = 6/group.
5.4 DISCUSSION

In the second experiment described in this thesis (Chapter four), it was reported that athletes who participated in a 3-wk training programme in which high intensity interval training sessions were commenced with low muscle glycogen concentration increased the maximal activities of several enzymes promoting endurance adaptations to a greater extent than athletes who began all training sessions with normal glycogen levels. As chronic training adaptations are thought to be the cumulative effects of repeated acute bouts of exercise (Hansen, et al., 2005; Hawley, et al., 2006), the present experiment sought to determine acute cell signalling responses to a single session of HIT commenced with either low- or normal muscle glycogen concentrations in an attempt to elucidate the potential mechanisms that might underlie the results observed in previous chapter in this thesis (Chapter 4). The main findings of the present study were that 1) AMPK phosphorylation was greater when HIT was commenced with low compared to normal muscle glycogen availability, and 2) despite the greater AMPK activation after intense interval training with low muscle glycogen availability, the localisation and phosphorylation state of selected downstream targets of AMPK (HDAC5, CREB) were unchanged.

AMPK is a member of a metabolite-sensing protein kinase family that functions as a metabolic 'fuel gauge' in skeletal muscle. During exercise, AMPK becomes activated in response to changes in cellular energy status (e.g. increased adenosine monophosphate [AMP]/adenosine triphosphate [ATP] ratio) in an intensity-dependent manner and serves to inhibit ATP-consuming pathways, and activate pathways involved in carbohydrate and FA metabolism to restore ATP levels. AMPK may also be involved in the adaptive response of skeletal muscles to endurance exercise training [for review see (Reznick and Shulman, 2006)]. Previous reports, largely from muscle
biopsy studies in untrained humans, demonstrate that activation of AMPK during exercise occurs in an intensity- and duration-dependent manner: there is little or no increase in phosphorylation until the relative exercise intensity is > 50-60% of VO\(_{2\text{peak}}\), whereas AMPK becomes progressively more phosphorylated with exercise duration. For example, Fujii et al. (2000) reported that AMPK activity was significantly increased in the vastus lateralis muscle following 20 min of cycling at 70% but not at 50% VO\(_{2\text{peak}}\). With respect to exercise duration, AMPK activity increases during the first 5 min of moderate (60% of VO\(_{2\text{peak}}\)) cycling with further rises observed after 30 min of exercise (Stephens, et al., 2002). AMPK becomes progressively more phosphorylated during prolonged (~210 min) low (45% VO\(_{2\text{peak}}\)) intensity cycling exercise (Wojtaszewski, et al., 2002) with these responses more closely coupled to muscle glycogen content than muscle AMP/ATP ratio.

In the current study, the HIT session was associated with a significant elevation of AMPK in all subjects, regardless of whether they commenced the session with low- or normal muscle glycogen levels (Figure 5.4A). However, AMPK phosphorylation was increased to a greater extent when HIT was commenced with low compared with normal muscle glycogen availability (Figure 5.4A). This greater AMPK activation occurred despite subjects attaining both higher absolute and relative power outputs when they commenced the HIT session with normal glycogen levels (Figure 5.3). In order to create conditions that are similar to previous study (Chapter 4), and to account for differences in the ability to train (i.e. relative intensity) with different levels of substrate availability, the subjects were deliberately instructed to cycle at their self-selected maximal effort during HIT sessions rather than ‘clamping’ the training intensity. Wojtaszewski et al. (2003) have previously reported that AMPK activity in resting human muscle
and the degree of activation during subsequent cycling exercise are dependent on the fuel status of the muscle cells (i.e., AMPK activity is elevated in muscle low in glycogen). In support of the current results where AMPK was found to be activated to a greater extent when intense exercise was perform in the face of low muscle glycogen availability, McBride, et al., (2009) recently provided first evidence that glycogen binding to the glycogen-binding domain on the AMPK β subunit allosterically inhibits AMPK activity and phosphorylation by upstream kinases. More importantly, removal of the outer chains of glycogen by phosphorylase (such as during exercise), thus exposing outer branches, renders inhibition of AMPK more potent (McBride, et al., 2009). Taken collectively these observations strongly support the notion that glycogen content in skeletal muscle modulates the AMPK response to contraction and also highlight the interactive effects of exercise intensity and endogenous fuel availability in inducing training adaptations in well-trained athletes.

In an effort to determine whether the increased AMPK activation when subjects performed HIT in the face of low muscle glycogen availability might contribute to the differences in training adaptation observed previously (Chapter Four), several downstream targets of AMPK were assessed. AMPK has been reported to phosphorylate and inactivate HDAC5 (McGee, et al., 2008b), which in turn leads to the removal of HDAC5 from the nucleus, allowing the myocyte enhancer factor 2 (MEF2) to bind and activate the PGC-1α promoter (Baar and McGee, 2008). In addition, PGC-1α is also known to have a CRE response element in its promoter region (Branvold, et al., 2008) while CREB has also been identified as the downstream target of AMPK (Thomson, et al., 2008). As such it was hypothesised that the increased in AMPK activation in response to intense training with low glycogen availability would result in concomitant increases
in the localisation and/or phosphorylation of some of these downstream targets of AMPK. This, however, was not the case: there were no differences in the localisation of HDAC5 and the phosphorylation state of CREB when subjects commenced intense exercise with either low or normal muscle glycogen levels (Figure 5.6). One explanation for these observations is that the magnitude of increase in AMPK phosphorylation was insufficient to specifically increase its activity towards HDAC5 and CREB. Alternatively, there may be other mechanisms by which chronic activation of AMPK increases mitochondrial enzyme activity, including but not limited to, AMPK effects on upstream stimulatory factor-1 (USF-1) (Irrcher, et al., 2008) and/or PGC-1α phosphorylation (Jager, et al., 2007) and/or localisation. Due to technical and muscle tissue limitations, these downstream signalling pathways of AMPK were not able to be measured for this thesis. Future studies should probably include alternative approaches (e.g. measurement of enzyme assays or mRNA expression) that may yield additional information regarding potential mechanisms by which AMPK exerts in effect on mitochondrial biogenesis. Nevertheless, total PGC-1α content was measured in the present study and was found to be unchanged by exercise in both groups (Figure 5.7). This result is in agreement with previous study in human subjects that reported PGC-1α was unchanged after 4 bouts of sprint cycling (Gibala, et. al.2009). However, as PGC-1α protein content does not represent its transcriptional co-activity, it cannot be determined whether or not completion of high intensity training in the LOW group transiently increased PGC-1α activity, thereby contributing to the changes in mitochondrial enzymes observed in previous study (Chapter Four).

Nevertheless, the more pronounced increase in AMPK phosphorylation observed in the present study does provide a strong mechanistic link between performing intense endurance training in
the face of low muscle glycogen availability and the subsequent increased in muscle glycogen storage capacity observed in previous study in Chapter 4, and others (Hansen, et al., 2005). In this regard, it is likely that the constant depletion of muscle glycogen during this nutrient-training strategy may have increased the body capacity to store muscle glycogen through higher AMPK phosphorylation. While the role of AMPK in mediating the adaptations in muscle glycogen storage capacity has not been directly proven in human, such phenomenon has been demonstrated consistently in animal studies (Barnes, et al., 2005; Holmes, et al., 1999).

The MAPK signal transduction cascade has been identified as a candidate system that converts contraction-induced biochemical perturbations in skeletal muscle into appropriate intracellular responses. The MAPK pathways include the stress-activated protein kinase p38 MAPK, which has been reported to be involved in mediating skeletal muscle adaptive responses to endurance training (Akimoto, et al., 2005; Fan, et al., 2004). Despite the higher AMPK phosphorylation in subjects who commenced HIT with low muscle glycogen concentrations, the phosphorylation state of p38 MAPK was not different between groups before and after the HIT session (Figure 5.4B). In contrast, Yu et al. (2003), have previously reported a significant increase in p38 MAPK after intense exercise in subjects similar to those recruited for the present investigation. Differences between results from the experiments of Yu et al. (2003) and the current study are hard to explain, although training intensity was ‘clamped’ in the former investigation and was markedly higher than the ‘self selected’ intensity chosen by the subjects in the present study. Furthermore, in that study Yu et al. (2003) observed a main effect for time (pre vs. post) but no interaction with training status. Not surprisingly given the lack of differences in p38 MAPK
signalling, the phosphorylation state of the selected downstream target of p38 MAPK (ATF2) was not different between groups (Figure 5.6C).

CaMKII has been implicated in skeletal muscle endurance training adaptation, with its degree of activation dependant on the prevailing exercise intensity (Chin, 2004; Rose, et al., 2006). In the present study, the phosphorylation state of the βm isoform of CaMKII was increased significantly only when the high intensity interval training was performed with normal muscle glycogen concentration (Figure 5.5). However, in contrast to a previous study by Rose et al. (2006) that reported an exercise effect on CaMKII-δ/γ isoform phosphorylation but not CaMKII-βm isoform, we were not able to detect any significant increase in the phosphorylation state of CaMKII-δ/γ isoform. Differences in results from the present investigation and that of Rose et al. (2006) may be related to the training status of the subjects and the absolute exercise intensities used in these studies. This increase in the phosphorylation of CaMKII-βm isoform is most likely due to the differences in training power output between the two groups of subjects. Nevertheless, when all the three CAMKII isoforms were quantified collectively, there were no effects of either treatment or time on the phosphorylation state of CAMKII.

The present study has focused on the early signalling responses to a single bout of HIT commenced with low- or normal muscle glycogen. While the findings in the present study strongly suggest that resting muscle substrate availability plays an important regulatory role in AMPK activation during subsequent exercise, one should not discount other factors as being important in modulating this response. For example catecholamine levels when exercise is undertaken with low muscle glycogen are higher than when the same intensity exercise is
performed with normal muscle glycogen stores (Hansen, et al., 2005), demonstrating that a higher stress response is elicited when training is commenced with low glycogen availability. When several bouts of moderate to intense exercise (50-75% VO$_2$ peak) are performed in succession, separated by 30-60 min of rest intervals, the secretion of growth hormone (Kaciuba-Uscilko et al., 1992; Kanaley et al., 1997), catecholamines, glucagon (Marliss et al., 1991; Kaciuba-Uscilko et al., 1992) and cortisol (Kaciuba-Uscilko et al., 1992) are progressively increased. However, not all studies demonstrate this effect and Bremner et al. (1977) have reported similar catecholamine, growth hormone and cortisol responses during two 30 min bouts of exercise at 50% VO$_2$ peak (Brenner et al., 1997). Ronsen et al. (2002) reported that plasma interleukin (IL)-6 and IL-1 receptor antagonist (IL-1ra) responses were significantly elevated when a second bout of exercise was performed only after 3 hr of recovery, an effect the authors attributed to muscle glycogen depletion. Arterial plasma FFA concentrations are also markedly elevated during exercise and subsequent recovery in well-trained subjects who perform steady-state exercise with low glycogen levels (Wojtaszewski, et al., 2003). This latter finding may be particularly relevant as raising plasma FFA concentration in the absence of exercise has been shown to induce mitochondrial biogenesis in skeletal muscle (Garcia-Roves, et al., 2007). Clearly the interaction of muscle fuel stores and the concomitant hormonal milieu during and after exercise play major roles in modulating the training response and subsequent adaptation and this is an area for future research.

In summary, the present study shows that high intensity interval training resulted in a significant increase in the activation of AMPK, with the magnitude of this increase being greater when training was commenced with low compared to normal muscle glycogen
availability. Despite the greater AMPK activation after intense exercise in the face of low muscle glycogen availability, the localisation and phosphorylation state of selected downstream targets of AMPK (HDAC5, CREB) were unchanged. Further studies are needed to identify the mechanisms responsible for the amplified training response observed when well-trained subjects commence intense cycling with low muscle glycogen availability.
Chapter Six

Summary and Conclusions
Summary & Conclusions

The primary aim of the studies undertaken in this thesis was to enhance our understanding of how nutrient availability in skeletal muscle interacts with endurance exercise to modify skeletal muscle adaptation in already well-trained athletes. Nutrient levels were manipulated by two different strategies: first by prescribing a high-fat, low-CHO diet, and secondly by manipulating the athletes training schedule. The subsequent training adaptation was studied by examining changes in whole-body metabolism and enzymatic activities as well as changes to selected cell signalling pathways that are related to both nutrient availability and training stimuli.

The first study (Chapter Three) employed a dietary periodisation model called “fat adaptation and CHO restoration” to investigate the mechanisms underlying shifts in substrate oxidation during exercise after such dietary intervention in well-trained athletes. Previous studies have shown that this dietary periodisation protocol enhances the ability to oxidise fat during submaximal exercise in well-trained athletes. As the multi-substrate enzyme AMPK has been implicated as the “metabolic sensor” in skeletal muscle, the hypothesis for this study was that fat adaptation and CHO restoration would result in changes in skeletal muscle fuel storage profile, and that these changes in fuel storage profile and would activate AMPK to induce the subsequent changes in substrate metabolism during exercise.

The first novel finding of this study was that fat adaptation and CHO restoration increased both lipid and CHO storage in skeletal muscle. It was discovered that resting AMPKα1 and –α2 activity was elevated after FAT-adapt, while the phosphorylation states of AMPK’s downstream target, ACC, tended to be higher at rest and was significantly higher after exercise.
during FAT-adapt. These results provided the first evidence of the mechanisms by which this dietary periodisation strategy serves to enhance rates of fat oxidation in trained individuals. In addition, while previous studies have suggested a role of muscle glycogen in regulating AMPK activity, the results of this experiment sheds new light for a role of muscle lipid status as a regulator of resting AMPK activity. The significant association observed between muscle TG levels and AMPKα2 activity provides valid support for this contention. In addition, a significant association between resting muscle glycogen concentration and basal AMPK α1 activity was also observed, further supporting the role of nutrient availability in modulating skeletal muscle training adaptation. Taken collectively, the results of this study suggest that fuel selection in skeletal muscle in response to exercise can be manipulated by diet and/or the interactive effects of diet and exercise training and such effects are mediated by AMPK.

Given the observed increased in resting AMPK activity after FAT-adapt, and that AMPK has been implicated in mediating mitochondrial biogenesis in response to exercise training, it seemed prudent to investigate if training in the face of low muscle glycogen availability would enhance training adaptation. Accordingly, the second study (Chapter Four) was undertaken to determine the effects of a cycle training programme in which selected training sessions were performed with low muscle glycogen content on training capacity and subsequent endurance performance, whole body substrate oxidation during submaximal exercise, and several mitochondrial enzymes and signalling proteins associated with enhanced endurance training adaptation in well-trained athletes. As well-trained athletes should already have maximised their training adaptation, the hypothesis of this study was that training adaptations would be
minimal, irrespective of whether the subjects trained with low or normal of muscle glycogen levels.

The major finding from this study was that when well-trained athletes undertook a 3 wk cycle training programme in which selected HIT sessions were performed with low muscle glycogen content, they were able to increase their capacity to oxidise fat while concomitantly increasing resting muscle glycogen concentration. Furthermore, several mitochondrial enzymes and proteins associated with enhanced endurance training adaptation, such as CS, β-HAD and COXIV were increased only in subjects who commenced exercise with low muscle glycogen stores. Interestingly, these adaptations were achieved despite the fact that the athletes were training at a lower relative intensity compared to athletes that performed their HIT sessions with normal muscle glycogen concentrations. In this regard, it appears that nutrient availability and associated metabolic stress should be taken into consideration as one of the factors that contribute to the overall ‘training stimulus’, which originally proposed to comprise mainly the inputs from the intensity, duration and frequency of exercise. Indeed, the results of this study suggest that compared with training daily, training twice every second day compromised high-intensity training capacity but augmented selected markers of training adaptation. Nevertheless, despite creating conditions that, in theory, should have enhanced endurance performance capacity, performance of a 1-h time trial undertaken after a 60-min steady-state ride was similar after once daily or twice every second day training.

The third experiment undertaken in this thesis (Chapter Five) was undertaken to elucidate the potential mechanisms underlying the enhanced adaptations seen with performing selected training sessions with LOW muscle glycogen concentration. In this regard, AMPK has been
proposed as the ‘metabolic fuel sensor’ in skeletal muscle and is activated during metabolic stress. Chronic activation of AMPK has also been reported to lead to mitochondrial biogenesis and associated increase in mitochondrial enzymes and proteins. Several putative signalling proteins such as HDAC5 and CREB have also been implicated as the downstream target of AMPK by which AMPK exerts its effects to induce mitochondrial biogenesis. p38 MAPK is another signalling protein that has been reported to be regulated by nutrient levels (i.e. low glycogen concentration) and has also been shown to play a role in mitochondrial biogenesis. Accordingly, the hypothesis of this study was performing intense endurance exercise in the face of low muscle glycogen concentration would result in higher of AMPK and p38 MAPK and some of their downstream substrates.

For the first time it was shown that performing intense endurance exercise in the face of low muscle glycogen concentration resulted in higher activation of AMPK, suggesting that the favorable adaptations seen in the previous study (Chapter Four) were likely to be mediated by AMPK. However, in contrast to the original hypothesis, the activation of p38 MAPK and CaMKII (all isoforms), and the phosphorylation and localisation of AMPK downstream targets was unchanged, indicating that either the activation of AMPK in this study is not strong enough to exert its effects on its downstream target, or AMPK might have exert its effects by another pathway/s.

The results from the experiments undertaken for this thesis show that nutrient availability can affect training capacity and is a powerful modulator of subsequent metabolic and skeletal muscle adaptation. In addition, the results also provide novel insights into the mechanisms by which
nutrient availability interacts with endurance training to modulate whole-body metabolism and skeletal muscle adaptation. Perhaps, the more exciting prospect is the findings from this thesis indicate that there is a role for ‘dietary periodisation’ strategies to be utilised to enhanced training adaptation. Rather than having athletes ingest a high CHO diet all year long, nutrient availability may be modified to promote intended training adaptations. For example, during the general preparation phase where the training goal is to build an aerobic base (i.e. to develop metabolic capacity), dietary carbohydrate intake may be deliberately reduced and dietary fat intake increased to promote the desired training adaptations (i.e. mitochondrial biogenesis). The negative effect of such strategy on training capacity should not be an issue as training volume is generally more important than training intensity during this phase of training. However, as training intensity becomes progressively more important, dietary carbohydrate content should be increased and dietary fat content should be decreased accordingly. Nevertheless, the optimisation of training using nutrient availability is still far from established and there are several questions that should be addressed by future research. First, in regards to fat adaptation and CHO restoration, it is not clear if the increased AMPK activity observed can indeed lead to augmented mitochondrial biogenesis. Should this be possible, studies should also be designed to explore how best to fit these nutrient-training strategies into macro- and micro-cycles of endurance training programmes. In addition, clear mechanisms linking fat adaptation to suppression of glucose metabolism, such as glucose transport, oxidation and enzymes linked to glycogen synthesis and breakdown have been important missing pieces of puzzle that might partly explain the observed adaptive responses and can possible serve to further optimise the protocol.
In the context of training once daily versus twice every second day, an important question that remains to be addressed is if such a training strategy would also resulted in an inhibition of CHO metabolism pathways (i.e. pyruvate dehydrogenase). Future research should also investigate the role of catecholamine and free fatty acid levels in modulating AMPK activation when exercise is undertaken with low muscle glycogen stores. Moreover, should technology be made available and accessible, it would be appropriate to examine if AMPK can indeed directly phosphorylate the transcriptional co-activator PGC1α and/or the upstream stimulatory factor-1 to induce the adaptations associated with this training strategy. While the results from the experiments in this thesis suggested that performing intense training in the face of low muscle glycogen availability can enhance the skeletal muscle adaptations profile, it would be interesting to know how low the muscle glycogen concentration has to decline before the beneficial adaptation of such training strategy is offset by the compromised training capacity.

In conclusion, the results from the experiments in this thesis provides novel information on the effects of nutrient availability and/or the interactive effects of nutrient and training on metabolism and skeletal muscle adaptation in well-trained athletes and the mechanisms associated with these adaptations. Continued discovery of the mechanism associated with the interactive effects of nutrient availability and exercise training should enable training programmes to be planned in a more scientific manner and the optimisation of training adaptations by manipulating nutrient availability may indeed be possible!
Chapter Six

References


